AMERICAN SOCIETY FOR HISTOCOMPATIBILITY AND IMMUNOGENETICS

ASHI Laboratory Manual

Fourth Edition

Volume I

Copyright © 2000. American Society for Histocompatibility and Immunogenetics. All rights reserved.

Editors

Amy B. Hahn, PhD, dip.ABHI
Geoffrey A. Land, PhD, HCLD
Rosemarie M. Strothman

Section Editors

Serology:

Cynthia E. Blanck, PhD
Donna L. Phelan, BA, CHS, MT(HEW)

Cellular:

Patrick W. Adams, MS, CHS
Lois E. Regen, MS, BA, CHS

Molecular Testing:

Debra Kukuruga, PhD, dip.ABHI
Harriet Noreen, CHS

Flow Cytometry:

Joan E. Holcomb, MS, CHS
Lauralynn K. Lebeck, PhD, MS, dip.ABHI

Quality Assurance:

Deborah O. Crowe, PhD, dip.ABHI

ASHI
Laboratory Manual
4th Edition

Table of Contents

Editors i

Preface ii

Table of Contents iii

VOLUME I: Serological Testing, Cell Mediated Testing, and Quality Assurance

I. SEROLOGY

A. CELL ISOLATION

Guidelines for Specimen Collection, Storage and Transportation I.A.1.1
Louise M. Jacobbi and Paula Blackwell

Principles of Cell Isolation: Overview of Current Methodologies I.A.2.1
Howard M. Gebel and Robert A. Bray

Density Gradient Isolation of Peripheral Blood Lymphocytes I.A.3.1
Brenda B. Nisperos

Augmentation with Monoclonal Antibodies (Lympho-Kwik ™) I.A.3.5

Isolation of Lymphocytes from Lymph Nodes and Spleen I.A.4.1
William M. LeFor

Immunogenetic Isolation of Lymphocyte Subsets Using Monoclonal Antibody-Coated Beads I.A.5.1
Julia A. Hackett and Nancy F. Hensel

Nylon Wool Separation of T and B Lymphocytes. I.A.6.1
Marilena Fotino and Arvind K Menon

Isolation of T Lymphocytes: A Quick Mini Method for Small Sample Sizes I.A.7.1
Afzal Nikaein

Rosetting as a Method for Separating Human B Cells and T Cells I.A.8.1
Dod Stewart and Sue Herbert

Isolation of Monocytes From Peripheral Blood Mononuclear Cells. I.A.9.1
Myra Coppage

Isolation of Endothelial Cells I.A.10.1
Nufatt Leong

Isolation of Granulocytes. I.A.11.1
Prema R. Madyastha

Assessment of Cell Preparations: A. Viability and B. Purity I.A.12.1
Mary S. Leffel

B. SERUM PREPARATION

Recalcification of Plasma I.B.1.1
Herbert A. Perkins, Nancy Sakahara and Zenaida P. Gantan

Absorption with Lymphocytes I.B.2.1
Gary A. Teresi and Anne Fuller

Extraction of Antibodies from Placentas I.B.3.1
Alan R. Smerglia

Inactivation of IgM Antibodies: A. DTT Treatment and B. Heat Inactivation I.B.4.1
Amy B. Hahn

Depletion of OKT3 From Serum I.B.5.1
Lori Dombrausky Osowski and Donna Fitzpatrick

General Concepts in Preparation of Monoclonal Antibodies I.B.6.1
Paul J. Martin

C. HLA CYTOTOXICITY TESTING

The Basic Lymphocyte Microcytotoxicity Tests: Standard and AHG Enhancement I.C.1.1
Katherine A. Hopkins

Serologic Typing of HLA Antigens by Monoclonal Antibodies I.C.2.1
Jar-How Lee and Jimmy Loon

Enhancement of MHC Antigen Expression I.C.3.1
Patrick W. Adams and Charles G. Orosz

Granulocyte Antigens and Antibodies. I.C.4.1
Mary E. Clay, Gail Eiber, and Agustin P. Dalmasso

Fluorochromatic Microgranulocytotoxicity I.C.5.1
Prema R. Madyastha

Monocyte Cytotoxicity I.C.6.1
Peter Stastny

The Use of Cultured Fetal Cells, Non-Lymphoid Tumor Cells and Fibroblasts for HLA Typing I.C.7.1
Marilyn Pollack

Anti-Idiotype Assay I.C.8.1
Elaine Reed and Nicole Suciu-Foca

Lymphocyte Crossmatch: Extended Incubation and Antiglobulin Augmented I.C.9.1
Patti A. Saiz and Cynthia E. Blanck

AHG Premixed With Complement: Streamlining for Protocols. I.C.10.1
Laura D. Roberts and Anne Fuller

Premixing of C' and AHG for Standardization of AHG T Cell Crossmatches I.C.11.1
Lori Dombrausky Osowski and Jeffrey McCormack

T and B Lymphocyte Crossmatches Using Immunomagnetic Beads I.C.12.1
Smita Vaidya and Todd Cooper

Interpretation of Cross Match Results I.C.13.1
Diane J. Pidwell

D. ELISA BASED ASSAYS

Crossmatches Using Solubilized Alloantigens I.D.1.1
Patrice Hennessy, Patrick Adams, and Charles Orosz

HLA Antibody Screening and Identification by ELISA Methodology I.D.2.1
Lori Dombrausky Osowski, Martin Gutierrez, and Beverly Muth

II. CELLULAR

A. CRYOPRESERVATION

Cell Preservation II.A.1.1
David F. Lorentzen

Cryopreservation of Lymphocytes in Bulk II.A.2.1
D. Michael Strong

Cryopreservation of Lymphoblastoid Cell Lines II.A.3.1
Soldano Ferrone

Cryopreservation of Lymphocytes in Trays II.A.4.1
Donna L. Phelan

B. PREPARATION OF CELL LINES

Growth of Lymphoblastoid Cell Lines and Clones II.B.1.1
Edgar L. Milford and Lisa Ratner

Preparation of B-Cell Lines II.B.2.1
Paul J. Martin

T-Cell Cloning II.B.3.1
Debra K. Newton-Nash and David D. Eckels

Propagation of Lymphoid Cells from Biopsies II.B.4.1
Adriana Zeevi

C. FUNCTIONAL ASSAYS

The Mixed Lymphocyte Culture (MLC) Test II.C.1.1
Eric M. Mickelson, Leigh Ann Guthrie, and John A. Hansen

HLA-Dw Typing II.C.2.1
Nancy Reinsmoen and Eric Mickelson

The Primed Lymphocyte Test (PLT) II.C.3.1
Nancy Reinsmoen

In Vitro Measurements of Cell-Mediated Cytotoxicity: Cytotoxic Effector Cells II.C.4.1
Sandra W. Helman and Malak Y. Kotb

III. QUALITY ASSURANCE

A. THE QUALITY ASSURANCE / IMPROVEMENT PROGRAM

Deborah O. Crowe

Overview III.A.1.1

Manual III.A.1.2

Forms III.A.1.5

The Quality Assurance Report III.A.1.5

Process Improvement III.A.1.6

Benefits of a Quality Assurance Program III.A.1.6

B. QUALITY ASSURANCE OF INFORMATION / DATA IN THE LABORATORY

Lori Dombrausky-Osowski

New Test Validation III.B.1.1

Patient Test Management III.B.1.2

Computer Validation III.B.1.4

Laboratory Data Maintenance III.B.1.4

C. FACILITIES AND ENVIRONMENT
Geoffrey A. Land
Physical Facilities III.C.1.1
Biologic and Chemical Hazards III.C.1.6
Radiation Hazards III.C.1.13

D. QUALITY CONTROL PROGRAM
Anthony L. Roggero and Deborah O. Crowe
Principle/Purpose III.D.1.1
Proficiency Testing III.D.1.1
Reagent Quality Control III.D.1.1
Complement Quality Control III.D.1.3
Anti-Human Globulin Quality Control III.D.1.3
Primer Quality Control for DNA Typing III.D.1.4
Probe Quality Control for DNA Typing III.D.1.5
Titration of FITC-Anti-Human IgG for Flow Crossmatching III.D.1.6
Equipment Maintenance III.D.1.6
Synthesis of Rare DRB1 Allele Sequences for Quality Control of SSOP III.D.2.1
Debra D. Hiraki, Shalini Krishnaswamy, Carl F. Grumet
Quality Control for DNA Contamination III.D.3.1
Jeffrey M. McCormack

E. REGULATORY AGENCIES
The Joint Commission on Accreditation of Healthcare Organizations III.E.1.1
Anne Belanger
ASHI – The HCFA Perspective III.E.2.1
Sandra Pearson and Esther-Marie Carmichael

IV. APPENDICES

A. CONTRIBUTORS

B. STANDARDS

C. HLA ALLELES AND EQUIVALENT SEROLOGICAL TYPES

Guidelines for Specimen Collection, Storage, and Transportation

Louise M. Jacobbi and Paula H. Blackwell

Purpose

In this chapter the principles and methods of specimen collection and their influence on the tests are discussed. Although specific to cytotoxicity testing for clinical transplantation with a focus on the pitfalls of using specimens from cadaver donors, the descriptions and recommendations made here are applicable to any specimen collected.

One of the major challenges for the laboratory scientist is the translation of basic scientific discoveries into diagnostic and therapeutic procedures that will be useful to clinical medicine. In the last four decades, immunology laboratory methods have become more refined and tuned toward clinical medicine, specifically, transplantation. Because of the increased specificity and sensitivity, transplant immunology is playing a major role in improving graft survival in transplant recipients and in assisting in the diagnosis of disease, ultimately improving the quality of care delivered.

A goal of the clinical laboratory is to improve the availability, accuracy, and precision of laboratory tests. An understanding of the methodology should permit the clinician to order appropriate tests and interpret their results. Therefore, the specimen used for testing is the basis for the ultimate quality of the final test results. The sample collection and specimen preparation, storage and transportation methods used for each assay will have an impact on those final test results.

Little has changed since the last issue of this manual relating to the basic guidelines and principles regarding the collection, storage and transportation of specimens for clinical studies. Although new technologies such as DNA based assays, antibody coated bead technology, and new applications of ELISA techniques are rapidly replacing and supplementing traditional cytotoxicity assays, the requirements for optimal specimen collection, storage, and transportation remain much the same. Lastly, regulations pertaining to the transport of all human specimens are being enforced and more closely monitored by courier services. Documentation and validation of each step of the testing and transport procedure of specimens has become an innate part of laboratory function and is necessary to ensure reliable, uniform information for the clinician's use.

Clinical success is the result of evaluating and executing procedures based on objective criteria and reporting results in a timely fashion, in a format that is easily interpreted. These include patient:

1. History
2. Physical assessment
3. Management
4. Laboratory values
5. Surgical procedure (specific to transplantation)
6. Documentation and data interpretation

These factors, individually and collectively, may affect the ability to obtain reportable laboratory results and at a minimum, can drastically extend the amount of time needed to obtain useful results. We all appreciate the necessity of evaluating the results from routine clinical laboratory tests. A typing laboratory's primary goal should, therefore, focus on methods of obtaining relevant, objective laboratory information to help provide a clear clinical picture of the patient or donor being tested.

Accurate, timely and reproducible clinical testing is directly related to the timeliness and care taken when procuring and transporting a specimen for testing. This is true in all clinical laboratory medicine and is particularly true in histocompatibility testing, where cell (lymphocyte) viability still plays a key role in many test procedures in general use.

The histocompatibility testing laboratory usually employs procedures for some if not all of the following (reviewed in the *AACHT Laboratory Manual*[7] and the *ASHI Laboratory Manual*[8]):

1. Hemagglutination for ABO blood grouping
2. Microlymphocytotoxicity testing for HLA Class I and Class II antigens (A, B, C, DR typing) antibody specificity identification, and crossmatching.
3. Cellular assays [e.g., Mixed Lymphocyte Reactions (MLR)]
4. DNA-based techniques [e.g., Polymerase Chain Reaction (PCR)]
5. Flow cytometry for antibody screening, crossmatching, and leukocyte phenotyping
6. ELISA for antibody screening

Many external events influence these laboratory assays and care should be taken to know what, when and how they may influence test results as they will relate directly to how you interpret the test results. The collection, storage and transportation of sufficient quantities of specimens, appropriately labeled and procured, are as essential as the procurement and retrieval of the organ itself and/or the medical treatment of the patient.

Communication and verified documentation of data are the keys to knowing what these events are and determining how to eliminate, alter or use them to evaluate test results. The information needed can be best obtained from the clinical personnel involved. They can tell you, for each patient, the particular medications, fluids and other circumstances which may affect test procedures, but you must provide them with the list of medications, fluids and circumstances which can influence the test procedures.

Most of the techniques employed for routine HLA typing are variations of the microlymphocytotoxicity test and the MLC assay. In recent years, the evolution of newer assays is stemming from the Enzyme-Linked Immunosorbent Assay (ELISA), Isoelectric Focusing (IEF), and DNA techniques (e.g., PCR). The reliability of microcytotoxicity testing depends upon the ability of the laboratory to obtain an adequate number of viable lymphocytes, which are free of contamination. This, in turn, depends on the quantity and quality of the samples(s) from which the cells, specifically lymphocytes, are to be isolated.

HLA typing and crossmatching must always be done prior to kidney transplantation. Most centers performing pancreas transplants also require typing and crossmatching of donor and recipient. A pre-transplant crossmatch is strongly recommended for any recipient who is pre-sensitized and is worthwhile for all organ transplantation.

Blood and other specimens for testing must be obtained in a clinically correct manner and under appropriate conditions as determined by the laboratory. Ideally, the specimen should be received immediately following its procurement. When this is not possible, several procedures can help maintain adequate viability and/or stability of the specimen to be tested. These procedures should be followed when the specimen must be shipped or testing must be delayed.

Specimen

1. Serum

a. Sources
   1) Peripheral blood (no anticoagulant).
   2) Peripheral blood (anticoagulated).

Clotted blood samples collected from potential recipients are submitted to the histocompatibility laboratory for ABO testing, PRA testing and antibody analysis, crossmatching with potential donors, autocrossmatching, and storage in the event further testing is requested. Clotted whole blood samples collected from potential donors are submitted to the histocompatibility laboratory for ABO testing and storage. When serum on clotted blood is required as in the crossmatch, be sure that an empty red top tube is used for collection. Clotted blood specimens must be obtained prior to treatment of the subject with anticoagulant. If a clotted specimen is not available, a specimen collected with anticoagulant may be recalcified to remove the fibrinogen from the plasma to yield serum. If the patient has some anticoagulant on board, the type and dose should be communicated to the laboratory so that the appropriate steps may be taken to convert the anticoagulant. Treatment with agents such as protamine sulfate often results in unsatisfactory tests.

b. Preparation
   1) If blood has not completely clotted by the time it is received in the lab, allow blood to clot in the original closed container. If the clot adheres to the top of the tube, dislodge clot by removing top of collection tube. If the clot remains attached to the top of the tube, gently dislodge clot from upper wall of tube by "rimming" with a wooden applicator stick.
   2) Centrifuge the blood for 10 minutes at 850 to 1000 Relative Centrifugal Force or gravities(G).
   3) Label a storage tube, preferably one with a secure screw top, with the name of patient or donor, date of specimen collection, a unique identification number such as the patient's hospital number or donor's UNOS number, and initials of the technologist transferring the serum to the tube.
   4) When centrifugation is complete, promptly remove the serum (or plasma if a tube containing an anticoagulant was received) to the previously labeled tube. If plasma is recovered, proceed to the "Recalcification of Plasma" procedure.
   5) To prevent bacterial growth a solution of 10% sodium azide may be added in a volume that will yield a final concentration in the serum of 0.1%. This is approximately 3 µl of the 10% solution per ml of serum.
   6) Store at 4 – 8° C until needed for testing or until packaged for transportation. Long term storage should be at a temperature of at least –70° C.

2. Lymphocytes

The proportions of T and B lymphocytes in human tissue are shown in Table 1 (ASHI Manual[8] and SEOPF Reference Manual[14]).

Table 1: *Lymphocyte Distribution in Human Tissue

Tissue	% T Cells	% B Cells
Peripheral blood	50-90	5-20
Lymph node	75	25
Lymph, thymus	80	15
Spleen	50	50

* Lymphocyte distribution varies among individuals and at different times in the same individual, therefore, the above percentages may vary. (Some laboratories have found a higher percentage of B cells in lymph nodes than is indicated above).

a. Sources
   1) Anticoagulated peripheral blood
      i. *Sodium heparin anticoagulant* – Sodium heparin is considered a suitable anticoagulant for HLA typing and cellular assays. Heparin is known to preserve cell viability up to 72 hrs, optimally to 48 hrs, especially if the sample is sterile. Heparin prepared from beef lung is preferable, and preservative-free heparin is recommended. Most procedures call for 25-50 units of sodium (Na) heparin per ml of blood to prevent clotting throughout the test. Vacuum tubes, usually green tops containing sterile crystalline sodium heparin can be purchased and are adequate for most testing procedures. In these tubes the number of units of heparin per ml of blood is lower. Often lithium heparin is substituted for sodium heparin. This is not universally acceptable as a substitute for histocompatibility testing.
      ii. *Acid citrate dextrose anticoagulant* – Acid Citrate Dextrose (ACD) is used as an anticoagulant in many typing laboratories today. One center may prefer solution A, and another solution B. Each is available commercially in vacuum tubes and you should make your laboratory's preference known to the specimen collectors
      iii. *Other anticoagulant* – Other anticoagulants such as ethylenediaminetetraacetic acid (EDTA), sodium citrate or sodium oxalate, are not recommended for HLA cytotoxicity procedures. Many of these agents are chelaters, which remove divalent cations (e.g., calcium), from the blood and interfere with complement activation in the complement-dependent cytotoxicity assays. If an assay is not complement dependent, these agents may be suitable. Which one and under what circumstances should be communicated to the collectors. Table 2 provides the types of assay, anticoagulant of choice, optimal storage time and preferred storage temperature for specimens.
      iv. *Clotted specimens* – Occasionally, due to improper collection, specimens for HLA typing will be partially or fully clotted. It is possible to recover lymphocytes from clotted blood samples if the blood is only a few hours old and has not been refrigerated. However, the lymphocyte yield, viability and stability are greatly reduced and the procedure is more time consuming.

Table 2: Specimen Collection and Storage Requirements for Various Assays

Procedure	Storage Anticoagulant (of choice) (preferred)	Storage Time* (optimal)	Storage Temp.
SEROLOGIC ASSAY (cell)			
Cytotoxicity	Na Heparin or ACD	<48 hrs	RT
Flow Cytometry	Na Heparin or EDTA	<24 hrs	RT
SEROLOGIC ASSAY (serum)	None	<72 hrs	4° C
		>72 hrs	–20 to –70° C
CELLULAR ASSAY (sterile technique required)	Na Heparin or ACD	<24 hrs	RT
DNA ISOLATION	EDTA or ACD	<48 hrs	RT or 4° C
Testing results are optimal when assay is performed ASAP after specimen is obtained			

   2) Lymph nodes
      The lymph node is the tissue of choice when typing cadaver donors in many laboratories. Lymphocytes from lymph nodes are generally only obtained from cadaver donors because a surgical procedure is required. The advantage of this source is that the cells can be obtained quickly and with little contamination of unwanted cells, i.e., red cells, platelets and granulocytes. Also, most lymph nodes yield a good number of lymphocytes, which have a higher ratio of B lymphocytes than peripheral blood, making the cells from lymph nodes ideal for HLA-DR typing.
   3) Spleen
      The human spleen, like nodes, can only be obtained by a surgical procedure. Lymphocytes from spleen are numerous and even higher in B cell content than any other source (up to 50% B cells).

b. Preparation
   1) Peripheral blood
      Freshly drawn anticoagulated venous blood is the usual sample submitted for HLA typing. Preparation of lymphocytes for HLA typing and crossmatching from peripheral blood samples involves the removal of other blood components, namely, red cells, granulocytes, monocytes and platelets. Lymphocytes are usually isolated from anticoagulated blood by a density gradient and/or immunomagnetic bead preparation to obtain cells for use in the various assays. Sterile blood is necessary for cellular assays but not for most serologic procedures. However, a sterile blood specimen is recommended to maintain lymphocyte viability longer than 24 hours. This also helps prevent contamination that would interfere or completely prohibit typing. Cooling the blood prior to delivery to the laboratory may make it more difficult to remove contaminating cells from a cell preparation. Exposure of whole blood to temperature extremes, even briefly, will compromise not only lymphocyte viability but also the ability to separate lymphocytes from other blood cells. Failure to maintain aseptic technique for any specimen can result in a drastic loss of cell viability. Most extended patient treatment will reduce the number and viability of lymphocytes.

The amount of blood needed depends upon the amount of testing to be done and the absolute lymphocyte count (white blood cell count x percent of lymphocytes). Five-ten ml of whole blood is usually adequate for HLA-A, B, C typing. Volumes of 10-40 ml may be required for HLA-DR typing, depending upon the need for T and B lymphocyte separation. Additional blood will be required if lymphocytes are needed for crossmatching and the amount will vary depending on the size of the potential recipient pool (if typing is for a cadaver donor) and the crossmatch technique(s) employed. If the laboratory has a policy of archiving specimens tested, the volume normally stored should be included in the order for specimen to be drawn.

2) Lymph nodes and spleen
The lymph nodes and spleen should be placed in complete medium, with antibiotics for sterility and an additional source of protein such as bovine serum. Be careful to avoid glove powder in the specimen preparation. The powder's fluorescence under a microscope may make the test difficult to read. B cells isolated from peripheral blood are not always sufficient in number or viability for B cell crossmatching with a large recipient pool. The collectors need to be aware that a larger volume of B cells may be needed in this instance and can be provided during the first hour of organ recovery by procuring a few lymph nodes before dissecting out transplantable organs. Generally, the same number of cells obtained from 30 ml of whole blood can be retrieved from a piece of spleen half the size of a pencil eraser (Be careful to avoid glove powder here also). Failure to maintain aseptic technique for any specimen and failure to provide supportive media for lymph nodes and spleen can result in a drastic loss of cell viability.

Reagents and Supplies

1. Phlebotomy supplies:
   a. Tourniquet
   b. Evacuated blood tubes(sodium heparin, ACD, EDTA, etc.)
   c. Needles
   d. Needle/tube holder
   e. 70% alcohol preps or Betadine pads
   f. Dry sterile gauze pads
   g. Biohazard waste container
   h. Biohazard sharps container
   i. Gloves
   j. Bandages
   k. Marking pen for labels
2. Specimen handling supplies:
   a. Approved shipping containers
   b. Transfer pipets
   c. Serum storage tubes
   d. Gloves
   e. Sterile specimen containers
   f. Medium for tissue storage
   g. Biohazard waste container

Instrumentation/Special Equipment

1. Centrifuges
2. Ice maker
3. Refrigerator
4. Freezer

Calibration

Follow manufacturers' instructions for calibration of temperature and centrifugal speed for equipment, i.e., centrifuges, refrigerator, and freezer.

Quality Control

1. Centrifuges should be inspected regularly to ensure proper safety, speed and performance. Refer to manufacturer's instructions for proper service intervals.
2. Storage medium should be tested for lymphocytotoxicity before being put into use for specimen storage. The methods used for testing should be the same ones routinely used for serological crossmatches.
3. The laboratory must maintain a system to ensure reliable specimen identification, and must document each step in the processing and testing of patient specimens to assure that accurate test results are recorded.[9]
4. The anticoagulant/preservation medium used must be shown to preserve sample viability, antigens, and distributions of markers/characteristics of cells tested for the (maximum) length of time and under all the specified storage conditions that the laboratory permits, on the basis of documented or published stability tests, between sample collection and testing.[9]

5. The laboratory must have criteria for specimen rejection and a mechanism to assure that specimens are not tested when they do not meet the lab's criteria for acceptability.[9]

Procedure

The nature of histocompatibility/immunogenetic testing mandates exposure by the clinician to specimens that have been obtained from high risk patients who may not have yet been tested for transmissible diseases. OSHA guidelines are quite clear on the handling, type of container, shipping container and protective wear for routine handling of specimens of this kind. It is best to assume that all specimens entering the laboratory pose a high risk (see Quality Control chapter). Every laboratory should develop a practice and policy for the handling of these tissues which protects the handler and the integrity of the specimen. Samples must be individually labeled as to the name, or other unique identification marker, for the donor and the date of collection.

1. Phlebotomy

Environment: Room should be clean with limited traffic and a calm atmosphere

a. Hands should be thoroughly washed and dried before putting on a new pair of disposable gloves.
b. Properly identify the patient or donor. Have the person lie down or sit in a chair with his arm supported.
c. Review the test requisition and select the appropriate evacuated tube(s) and holder system or correct syringe and tubes for the assays ordered.
d. Apply the tourniquet well above the elbow.
e. Ask the patient to make a fist and open and close his hand a few times to better distend the veins in the arm.
f. Choose the venipuncture site by palpating the area below the tourniquet. Prolonged use of the tourniquet, even for 60 seconds, can falsely elevate the concentration of many blood constituents.
g. Clean the intended puncture site with 70% alcohol or Betadine swab. Allow to dry.
h. Select either a 20 or 21 gauge needle for the veins of the forearm or 25 gauge needle if a vein in the wrist, hand, or foot must be used. Wrist, hand, or foot veins should only be used after the arm veins are found to be unsuitable.
i. Enter the skin and vein in a single motion with the needle held bevel side up and pointing in the same direction as the path of the vein. Hold the syringe or evacuated tube at a 15° angle to the skin.
j. If an evacuated tube is used, the tube should be carefully pushed into the holder so that the tube cap is punctured with the inside needle and the blood is allowed to enter the tube. When multiple specimen collection tubes are used, each tube should be gently removed from the holder and replaced with another one. The tubes with anticoagulants should be mixed with one hand while waiting for another tube to fill with blood.
k. Remove the tourniquet once the blood begins to flow freely in the tubes or syringe.
l. After tourniquet release and collection of the appropriate tubes of blood, the needle should be withdrawn quickly. Immediately, apply gentle pressure to the venipuncture site with dry, sterile gauze to stop the bleeding. The arm may be kept straight or bent at the elbow. Raising the arm while applying gentle pressure to the venipuncture site may decrease the bleeding time.
m. If a syringe has been used, quickly remove the needle and immediately transfer the blood into the appropriate tube(s). Immediately cover and gently mix the tube by inverting 10-20 times if an anticoagulant is being used to prevent clotting.
n. Carefully label all tubes with the patient or donor's name, date, time, collector's initials, and any other information according to laboratory policies.
o. Dispose of needles in a special, sturdy disposable container to be appropriately destroyed. Never dispose of needles in a wastebasket that is accessible to other patients.
p. Before leaving the patient, check the venipuncture site to make sure that the bleeding has stopped and that the person is not experiencing any discomfort or anxiety. A small dressing may be applied, mainly to prevent a bloodstain on the clothing.

2. Procurement of Lymph Nodes

Environment: If procurement is not at the time of organ recovery, the room should be clean with no traffic. Site should be scrubbed with betadine and allowed to dry. Excision site should be covered with sterile drape. Use only sterile instruments and close the excision site with sterile suture. Usually only 2-3 lymph nodes are recovered.

During the organ recovery procedure the surgical team should excise 15-20 well-defined lymph nodes from the donor. The lymph node will sink when placed in the medium. Nodes from the mesentery are very difficult to identify if left in the mesenteric mass for any length of time. They take longer to remove and identify, and will delay testing. It is worth the time for the surgeons to cleanly dissect nodes in the operating room (OR) as it can shorten the cold ischemic time (CIT) of the organ(s) to be transplanted. In the OR usually 5-10 minutes is needed while in the lab it may take as long as 30 minutes to identify and isolate usable lymph nodes. Failure to maintain aseptic technique for any specimen and failure to provide supportive medium for lymph nodes can result in a drastic loss of cell viability. To reduce the overall time needed for testing, one can request, at the time of the procurement surgery, that the lymph nodes be removed first. When done prior to surgical removal of the organs, this procedure must be carried out under optimal conditions to protect the donor from pathogens prior to donation. With a multi-organ donor, the operating time can take 4-5 hrs and the collection of a few nodes at the beginning of the case would give the laboratory a head start. The CIT of an organ can be shortened if the typing is performed either pre-recovery or while the retrieval team

is in the operating room. Because it takes longer to isolate lymphocytes from peripheral blood than from lymph nodes, some advance planning may be in order. For instance, when lymph nodes can be obtained within one or two hrs after the peripheral blood, it is often faster and more effective to use the later-procured lymph nodes (since the isolation technique is faster). If organ recovery is imminent or if there is a possibility of obtaining nodes, one should obtain and transport both the peripheral blood and lymph nodes at the same time to the typing laboratory. By doing this, no time will be lost and the laboratory will be spared extensive and expensive effort. This effort requires communication and coordination with the surgical recovery team.

3. Procurement of Spleen

Environment: Recovery is always in an operating room since spleen is recovered after removal of all organs to be transplanted.

Because of the many B cells, the spleen should be placed in complete medium, with antibiotics for sterility and an additional source of protein such as bovine serum. To promote cell viability when procuring spleen, section it into small 2 cm squares so that the cells within can be exposed to the nutritive media. Most lymphocytes are found in patches, which lie close to large blood vessels within the spleen. The entire spleen should be taken, if possible, divided into sections and samples shipped to the back-up recipient centers.

4. Specimen Storage

Because viable cells are necessary for HLA typing by cytotoxicity methods, crossmatching and MLR's, non-specific death of lymphocytes by aging or other factors, which may occur after collection rapidly reduces the likelihood of a successful assay. Some specimens, such as serum and extracted DNA, can undergo greater stresses without negative consequences. In general, if storage conditions are optimum for lymphocytes, they will be adequate for red cell and/or serum assays since the lymphocyte is the more fragile tissue. If the whole blood, lymph node, or spleen sample is not handled properly, the likelihood of a successful typing by cytotoxicity is in jeopardy.

At a minimum, specimens should be retained in a laboratory until all procedures are reported out and there are no questions relating to results. A reasonable policy would be 72 hours after testing of routine specimens.

a. Peripheral blood
   1) Anticoagulated whole blood samples kept at room temperature (RT), up to 72 hrs, usually yield viable lymphocytes.
   2) If testing will be delayed, centrifuge the samples for 10 minutes at 850 – 1000G.
   3) Remove the plasma and discard or transfer to an appropriately labeled storage tube.
   4) Transfer the buffy coat to a tube containing an equal part of supplemented medium. (Alternatively, the entire whole blood sample may be transferred to another, larger tube containing supplemented medium).
   5) Store at 4 – 8° C until further cell isolation is indicated.

b. Lymph nodes and spleen sections.
   Lymphocytes from lymph nodes are more fragile than those from blood. This may be due to the higher percentage of B cells present in nodes and because B cells die more easily than T cells. To ensure viability and stability, lymph nodes should be procured aseptically and immediately placed in a complete medium such as RPMI 1640, Eagles or minimum essential medium (MEM) supplemented with a protein source such as Bovine Serum and with antibiotics. The container should be kept cool. Freezing temperatures must be avoided. Never store lymph nodes in saline (no nutrient). Although sterility is not necessary, for HLA typing, it is recommended in the event testing is delayed to aid in the prevention of bacterial contamination

5. Transportation

Despite all efforts, lymphocytes, once removed from the body, will die at a certain rate. Therefore, expedient and proper delivery of specimens to the laboratory is absolutely necessary. For assays requiring viable lymphocytes for dependable results, the two most important variables associated with loss of viability, are time and extremes of temperature. Any transported tissue is subject to both variables. Several measures are effectively used to reduce this loss during transportation.

a. The procedure for preparing containers to send to distant areas is as follows:
   1) Inspect sterile tubes with sodium heparin or ACD for blood specimens and sterile vials with culture medium for tissue specimens, as described. (Table 6)
   2) If you are the donor typing laboratory and have viable cell prep, make every effort to send it. Receipt of a viable cell prep will keep to a minimum the total CIT on the organ to be transplanted (Figure 1).
   3) Place the tubes in a Styrofoam container if the specimens are not being shipped with the donated organ (e.g., back up specimens to another center), bind them together or otherwise prevent them from hitting each other in transit (Figure 2).
   4) With the labeled specimens, include a note explaining any extenuating circumstances of donor history or include a copy of the donor's hospital chart.
   5) Include an emergency phone number for the transport company on the outside of the package.
   6) Send the package air express to the laboratory, which agreed to type samples. Be sure to let them know when and how the sample is coming. Transportation of specimens should be accomplished in the most efficient manner. Cost should be taken into consideration. Each case needs to be decided based on the merits of the options.

7) Use of a routing slip identifying each handler is the best means of tracking and distributing specimens. This is especially true and necessary when obtaining and testing specimens for parentage testing, forensic procedures, or if you are handling specimens from patients who may have a transmissible disease.
8) Take any precautions necessary to avoid exposing tissue to temperature extremes.
9) Avoid having package sitting on a hot loading dock.
10) Avoid putting specimen directly on ice.

b. SPECIMENS THAT ARE NOT APPROPRIATELY LABELED CANNOT BE PROCESSED BY THE LABORATORY. (This policy must be made clear to all collectors and clinicians).
c. THE SAME CARE MUST BE USED WHEN TRANSPORTING TYPING MATERIAL AS USED WHEN TRANSPORTING AN ORGAN.
d. When packaged with the organ:
   1) All blood tubes, vials and containers with donor tissue and specimens for testing must be properly labeled with the individual's identification and time and date of collection.
   2) The contents must not be frozen, dehydrated, exposed to water or fixatives. Natural salinity of the medium surrounding the tissue may cause freezing if the container is placed directly on ice.
   3) Lymph nodes and spleen should be packaged separately for quick access.
   4) Containers for nodes and spleen sections should be large enough to accommodate the material in supportive media. The containers should be filled with medium so that there is no chance of the tissue being without nutrient. Antibiotics in the medium will prolong sterility and thus, viability.
e. Lymphocytes from nodes and spleen appear not to be as adversely affected by refrigeration (4° C) as do those from blood, but care must be taken to avoid freezing the tissue. Figure 1 is a schematic of one method of packaging typing specimens with the organ, Figure 2 is a schematic of a method for separate packaging both using UNOS guidelines.

Figure 1. Packaging of Specimens with Organ

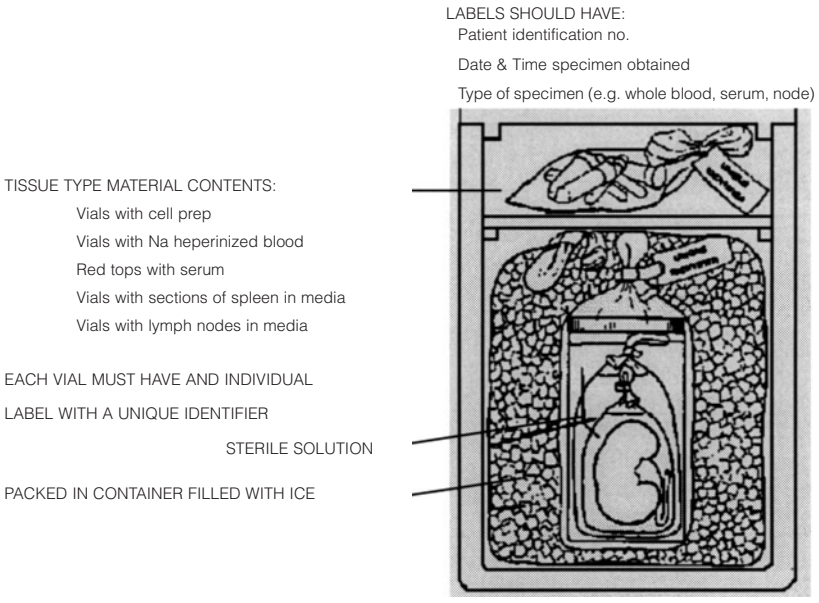
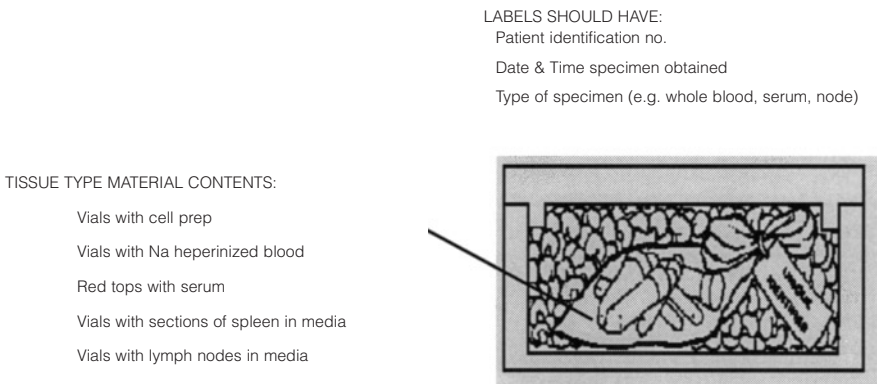

LABELS SHOULD HAVE:
Patient identification no.
Date & Time specimen obtained
Type of specimen (e.g. whole blood, serum, node)
TISSUE TYPE MATERIAL CONTENTS:
Vials with cell prep
Vials with Na heperinized blood
Red tops with serum
Vials with sections of spleen in media
Vials with lymph nodes in media
EACH VIAL MUST HAVE AND INDIVIDUAL
LABEL WITH A UNIQUE IDENTIFIER
STERILE SOLUTION
PACKED IN CONTAINER FILLED WITH ICE

Figure 2. Separate Packaging of Typing Specimens


LABELS SHOULD HAVE:
Patient identification no.
Date & Time specimen obtained
Type of specimen (e.g. whole blood, serum, node)
TISSUE TYPE MATERIAL CONTENTS:
Vials with cell prep
Vials with Na heperinized blood
Red tops with serum
Vials with sections of spleen in media
Vials with lymph nodes in media
EACH VIAL MUST HAVE AND INDIVIDUAL
LABEL WITH A UNIQUE IDENTIFIER

6. Documentation

Communication between the laboratory and the specimen collectors concerning history and any influencing factors is vital for accurate specimen documentation. Complete documentation will assist in the accuracy and timeliness of the test results. For regional and/or national sharing of cadaver donor organs (UNOS Standards and Policies[6]) donor information regarding the tissue or clinical condition of the donor should be sent routinely to the laboratory which receives a specimen. Such data is needed for confirmation of ABO and HLA typing and screening results. A hard copy of ABO typing, HLA typing, and crossmatching results should be sent with the specimens when sharing an organ.

a. Specimen reception

All samples should be entered into a log as they are received into the laboratory. Documentation in the logs varies depending on the type and needs of the laboratory. Much of the information is also needed to accurately complete registries for computerized data collection systems. Information as to the time samples are drawn may be critical for determining if transfusions or drug therapy has affected HLA tests or clinical assays used to evaluate a potential donor. Most laboratories keep the following data in some form:

1) Specimen history: patient name, identifier number, date and time received, volume, handlers
2) Specimen origin: signatures of handlers, date and time of transfer, date of log-in, aliquot disposition (tests performed, amounts stored)
3) Specimen storage: date, location and amounts
4) Specimen use: tests performed, date of testing, archived, research, and ordering physician
5) Specimen reporting: date, to whom and form of report (hard copy, verbal or fax)

b. Factors affecting volume of sample needed

1) The amount of blood requested will vary in situations in which the lymphocyte subset levels may be altered. Laboratory indication of this can be determined by:
   i. A recognized increase or decrease in the WBC with a differential which may cue a technologist to go directly to the spleen for cells rather than spending time trying to obtain cells from a whole blood specimen.
   ii. Immunoglobulin abnormalities.
   iii. A positive serology with presence of antibody to HIV.
2) Lymphocyte isolation problems can occur when patients have a high granulocyte count or are leukopenic. The clinical situations which may be present with these variances are:
   i. Lymphadenopathy.
   ii. Viral, fungal or protozoal infections.
   iii. Repeated infections.
   iv. AIDS or AIDS-related disorders.
   v. Immunosuppression.[2]
3) Effect of transfusions of blood products

   When massive amounts of blood are administered to patients or potential donor's extraneous reactions may be produced which may make interpreting typing results difficult if not impossible. Blood samples from transfused patients and donors should be collected at least 24 hrs after the last transfusion to reduce the possibility of typing the cells which were transfused. When possible, IT IS PREFERABLE TO TISSUE TYPE PRIOR TO ANY TRANSFUSION and as early in the patient's or donor's hospital course as possible. Alternatively, use of pre-recovery inguinal lymph nodes obviates the problems due to transfusion.
4) Effect of donor management and history.

   Information about donor medications, diagnosis, and clinical status may warn to expect unusual conditions. Table 3 is a checklist containing frequently needed and useful information.
5) ABO typing and HLA typing.

   It is highly recommended that a hard copy of the ABO typing and HLA typing results be on hand in the typing laboratory before either the recipient's or donor's information is entered into the UNOS program. This important quality assurance policy has been proven to prevent improper transplantation of organs, which were mislabeled or mixed up during testing or transportation.
6) Test Reports.
   i. Test results should always be reported out in hard copy to the physician ordering the procedure. The report form used should be easily interpreted or have a section on it that interprets results for the client. If a verbal report is requested it should only be released to those authorized by the physician ordering the procedure.
   ii. If you use a facsimile report to clients, always follow it with the original, hard copy. Also, the fax face sheet should be addressed to the authorized representative of the person who ordered the testing. Many laboratories use a cover note, which states that the information contained is privileged, and exempt from disclosure under the law. It also states that copying any part of the report is strictly prohibited.
   iii. Laboratories which are involved in testing for clients who require strict confidentiality (parentage, forensic, sexually related or transmitted disease) should have a written policy regarding the special handling of information by laboratory personnel (e.g., faxing or copying this information may be illegal in some states without the written permission of those involved). Confidentiality cannot be maintained over an open phone line and the use of this form of data transfer should be limited and may be prohibitive in

some circumstances. It is wise to seek legal counsel and know the laws regulating handling of sensitive information within your state as well as your company policy when developing in-house policy.

iv. CLIA 88 mandates archiving of laboratory reports for a minimum of two years. Every laboratory should have a written policy to the length of time, contents and type of storage to be used for all its records.

Table 3: Donor Information Checklist

Name	Culture reports
Unique identification number	HBs Ab test results
*UNOS number	HLA at donor lab
Hospital	Cell prep. source and amount
Date and time of specimen products with dates given	Medication and blood
Age, sex, race	*Which organ available
*Date and time organ removed	HIV test results
Referring physician	CMV test results
WBC & differential	Diagnosis and history
Length of hospital stay	ABO (hard copy)
*INFORMATION UNIQUE TO CADAVER ORGAN DONATION	

Calculations

Not applicable.

Results

Not applicable.

Procedure Notes

1. **Validation Procedures**
   Each essential task or step of any process should be validated. In the case of specimen preparation and transportation both time and temperature are key elements for successful typing as well as handling and specimen preparation. Where specifications are essential, a validation process should be implemented.

2. **Treatment Effects**
   Lymphocytes have a certain normal variability in their expression of HLA antigens, particularly DR antigens. This variability is exacerbated by certain treatments such as systemic steroid and immunosuppressant medication, by trauma, by certain diseases, and by transfusion. While transfusion may not technically alter antigen expression, the lymphocytes of a transfused subject may be a mixture of cells from the subject and the blood donors. Consequently HLA test results may not be representative of the patient's or donor's antigens.

3. **Quality of Blood to Draw**
   The amount of blood required to perform HLA-A, B, C and DR typing will vary somewhat among laboratories. Therefore, it is essential to provide information regarding your laboratory's requirements to those responsible for specimen procurement. Normally, 20-60 ml of blood is adequate for HLA-A, B, C, and DR typing and 50-100 ml of blood is sufficient for most MLR procedures (depending on the number of patients being tested and the amount of testing to be done at the same time). By most isolation procedures, each ml of blood, from individuals with a normal blood profile, should yield about 1 x 10^6 lymphocytes. Table 4 gives the approximate number of lymphocytes found in peripheral blood from normal individuals.[4] A larger volume of peripheral blood may be required from very sick patients or potential cadaver donors. A basic guideline for the amount required can be obtained from their white blood cell count and differential.

Table 4: White Blood Cells (WBC)/Differential

WBC Count	**4-11,000/mm^3**
Basophils	0-2
Eosinophils	0-3
Monocytes	2-6
Lymphocytes	20-45
Neutrophils	50-70

The amount of blood needed for typing a pediatric patient depends upon the reason for testing. If the patient is the recipient, knowledge of the patient's WBC count would be helpful in determining the volume of blood needed. If the subject is a cadaver donor, the size of the potential recipient pool must be taken into account. 60-100 ml of blood can easily be collected from an arterial line (of an adult donor). Since the lymphocyte count is usually higher in children, 25-40 ml is generally adequate for one attempt at HLA typing and preliminary crossmatching.

Care should be taken in drawing the specimen, particularly in pediatric or older patients, to prevent cell lysis and cell shearing. This can best be done in these patients by using a large bore needle and/or a syringe rather than a vacuum tube to obtain the specimen. Hemolysis has been known to interfere with some chemistries. In HLA typing, cell lysis may cause difficulties in cell separation and lymphocyte testing manifested as false positives or high background. When a specimen must be transferred from one container to another, aseptic technique should be adhered to; a large bore needle should be used; and full force of the vacuum should be avoided by sliding the specimen down the side of a vacuum tube just below the stopper.

4. Type of Specimen

Tissue specimens of choice vary from laboratory to laboratory. If you prefer spleen over lymph node it is best to educate your recovery team. The advantage of using peripheral blood and lymph nodes over spleen is time. They can both be obtained prior to surgical recovery of the organs. Peripheral blood can be obtained by any good phlebotomist. A lymph node requires surgical intervention but may be obtained prior to organ recovery.

The disadvantage to peripheral blood is the lack of expression of some DR antigens by some techniques. This largely has been overcome with newer cell separation and varied incubation techniques. Table 5 is a summary of the factors mentioned and the effect they can have on the condition and number of circulating blood lymphocytes.

Table 5: Factors Affecting Peripheral Blood Cells

Factors	Effect
Age	Decreasing number of lymphocytes with increasing age
Trauma respirator	Increasing proportion of polymorphonuclears (PMNs), increasing difficulty of lymphocyte isolation
Steroids	Decreasing lymphocyte HLA antigen expression, decreasing lymphocyte viability
Cytotoxic drugs	Decreasing number of lymphocytes, decreasing lymphocyte viability
Temp <2° C or >40° C	Decreasing ability to isolate lymphocytes from peripheral blood
>4° C	Decreasing cell viability in lymph nodes and spleen
Microbial contamination	Decreasing cell viability interferes with test interpretation; biohazard
Time	Decreasing viability with increasing time
Transfusion	May interfere with test interpretation
Needle bore size	Too small needle bore size can decrease number of viable lymphocytes
Vacuum tube	Too great a vacuum can cause cell lysis, decreasing cell viability

5. Pre-Recovery Typing

Delay in placing the organ can be reduced to a minimum if pre-recovery typing is performed on potential donors. Laboratories can provide this service if the previously mentioned information is obtained and those responsible for procuring specimens are instructed as follows.

a. Do not cool anticoagulated blood. Keep at RT until delivered to laboratory for testing.
b. Collect more (30-100 ml) anticoagulated blood than normally required.
c. Use appropriate anticoagulant (Table 2).
d. Provide the technologist with as much time as possible to perform assays.
e. Provide the technologist with white blood cell count and differential.
f. Provide the technologist with information about events that might alter technique used.

6. Speciment Needs and Requirements

Laboratories should provide their specific needs and requirements for each test procedure to their client. Table 6 is a summary of type, volume and storage requirements for most specimens required for a cadaver donor.

Table 6: Specimens

Specimen	Collect	Volume	*Storage	Transport
WHOLE BLOOD	Sterile tubes	40-120 ml adult, 25-60 ml pediatric, varies according to condition of donor and number of recipients to be tested	Na heparin, preservative-free or ACD (the amount of anti-coagulant in tubes is calculated for its size – each filled and inverted for proper anti-coagulation)	Tubes in styrofoam mailers; insulate from extreme heat, cold and breakage
SERUM	Same as whole blood	10-20 ml adult, 3-5 ml tube pediatric	Empty vacuum	Same as whole blood
LYMPH NODES	Sterile containers to which support medium has been added	3-5 for preliminary testing, **15-20 for complete testing. ALL NODES SHOULD BE WELL DEFINED AND DISSECTED FROM MESENTERIC MASS	Culture medium with antibiotic (RPMI 1640, MEM, Eagles)	Place tape over top to prevent accidental opening and place in ice slush to maintain at 4° C
SPLEEN	Same as nodes	Entire spleen, 2 cm squares	Same as nodes	Same as nodes

* DO NOT FREEZE. DO NOT ALLOW TO WARM.
** This allows the additional nodes needed for backup crossmatching.

7. Estimated Testing Time

The time period from the moment samples are collected until test results are reported will vary somewhat among laboratories. The primary factors that determine how much time is required are the condition of the test materials, the kind and number of tests requested and the number of patients to be tested. For example, a blood group O donor takes longer to test than other donors because of the greater number of patients that must be screened. Several patients may be crossmatched with one donor in the same amount of time as one, if all recipient specimens are available in the laboratory at the same time. When serum for additional crossmatches must be retrieved, titered and plated, the crossmatch will take more time. Impure preparations, marginally viable cells, and other variables will affect the amount of time required for testing in the laboratory. Those other variables include the speed with which different technologists work, the accessibility of facilities, the effectiveness of communication with coordinators, and the number of interruptions. These factors are usually consistent within a laboratory and can be anticipated. A general guideline of test times for one technologist is given in Table 7. The times given include cell preparation and are appropriate if no unusual problems develop. Each laboratory will have overall adjustments to these times.

Most of the tests can be run simultaneously, so that the tests with shorter duration can be included in the same time frame. Tests started at the same time are much easier for the technologist to manage. It is usually the rule, and not the exception, to encounter at least one problem that will increase the time or completion of testing when the laboratory is working up a cadaver donor.

Table 7: Time Estimates for Tissue Typing

Test	Time	Average
HLA-A, B, C typing	3-4 hrs	4-8 hrs
HLA-DR typing	3-6 hrs	
Regional Screen	3-4 hrs	3-6 hrs
Crossmatch Final	*3-6 hrs	
	Total Average:	7-14 hrs

* For 1-2 recipients whose samples are readily available only. When factors outside the laboratory's control are involved, such as delays in recipient selection, travel time of recipient to center, testing of up to 20 recipients, all of these may extend this range up to double the amount given.

8. Archived Specimens

Many laboratories archive specimens for future use. If you have such a practice it should be written in your policy and procedure manual. If longer storage is required a separate policy should be written for handling and length of time storage is needed. If frozen cell specimens are stored, the time, freezing medium and freezing process should be documented.

Limitations of Procedures

Obtaining and typing lymphocytes from some subjects can present special problems to the laboratory, particularly since the testing must be done as quickly as possible. While these procedures are easily performed using lymph nodes and spleen many problems can be encountered when using peripheral blood from the compromised patient or cadaver donor.

Testing of cadaver donors prior to organ recovery is desirable. However, unless special surgical procedures are initiated to obtain lymph nodes, peripheral blood may be the only tissue available. Some problems and their possible solutions are discussed below.

To perform histocompatibility tests which yield reliable results, the histocompatibility laboratory must isolate an adequate number of donor T and B lymphocytes, which are viable and free of contaminants (other cells and microorganisms). The number and condition of these lymphocytes can be affected by a number of intrinsic and extrinsic factors. The various problems which have been observed include: (1) a reduction in the total number of lymphocyte subsets; (2) an alteration in the relative proportions of the various types of WBC's; (3) a reduction in lymphocyte viability; and (4) an alteration in the reactivity of lymphocytes in the histocompatibility test. The absolute number of circulating lymphocytes and the relative proportions of various types of WBC's can be affected by the (past and current) condition of the patient being tested.[4] Age, gender, use of drugs, alcohol or tobacco, current infections, medical conditions and injury may all alter the distribution of WBC's in the circulation.[5] The single most important controllable factor affecting lymphocyte viability is the handling of the specimen, including the environment in which the specimen is maintained during transit to the laboratory and the amount of time which transpires between sample collection and delivery to the laboratory.

Pre-recovery typing of donors from blood samples has been successful in many laboratories under certain conditions. Administration of steroids leads to fewer lymphocytes and an increased number of granulocytes and platelets in peripheral blood. Therefore, it is recommended that samples for typing be collected before administration of steroid therapy, if possible, or at least several hrs after steroids have been reduced. When Decadron or Cytoxan has been administered, modification of the standard techniques is almost always necessary. It is advisable to request pre-recovery of a lymph node if it is known that a cadaver donor has been administered high dose, long term steroid therapy (e.g. three doses or more of Decadron greater than 10 mg per dose).

For cadaver donors, it is UNOS policy that a pre transfusion specimen be tested for HIV (UNOS Standards and Policies[6]). Remember that multiple transfusions can alter the ABO typing of a donor, cause false negative results of serological tests for infectious diseases, and create coagulation problems as well as interfere with typing results.

Many laboratories have experienced that subjects on systemic steroid and immunosuppressant medication are difficult to type for HLA-DR. For reasons not clearly understood, it appears that the HLA-DR molecules are either less well expressed, suppressed or modified so that reactivity of B cells with HLA-DR antisera is diminished. It has also been shown that lymphocytes from lymph nodes and spleen from some cadaver donors have detectable DR antigens; but the DR antigens of their circulating B cells do not always express themselves. The use of longer incubation times during this test procedure can sometimes eliminate this problem. The delay and the need for a longer incubation time should be conveyed to the clinicians, when additional time is required. More frequently now, the problem of less than optimal B lymphocytes for DR typing is overcome by using molecular techniques.

References

1. Becan-McBride K and Ross DL: *Essentials for the Small Laboratory and Physician's Office*. Year Book Medical Publishers, Inc., Chicago, 1988.
2. Burton RC, Ferguson M, Gray M, Hall J, Hayes M and Smart YC, Effects of age, gender and cigarette smoking on human immunoregulatory T-cell subsets: Establishment of normal ranges and comparison with patients with colorectal cancer and multiple sclerosis. In: *Diagnostic Immunology*. MF Lavia, ed., Alan R. Liss, Inc.; New York; 1:216, 1983.
3. Davidson I and Henry JB, *Clinical Diagnosis by Laboratory Methods*. W.B. Saunders Company, Philadelphia, 1984.
4. Giorgi JV, Lymphocyte street measurements; significance in clinical medicine. In: *Manual of Clinical Laboratory Immunology*, 3rd Edition; NR Rose, H Friedman, JL Fahey eds.; American Society Microbiology, p 236, 1986.
5. Green DR and Faist E, Trauma and the immune response. Immunology Today, 9:253, 1988.
6. UNOS Standards and Policies; UNOS, Richmond, 1992.
7. Zachary AA and Braun WE: *AACHT Laboratory Manual*, 2nd Edition, American Association for Clinical Histocompatibility Testing, 1981.
8. Zachary AA and Teresi G: *ASHI Laboratory Manual*. American Society for Histocompatibility and Immunogenetics, Lenexa, 1990.

ADDITIONAL REFERENCES

9. ASHI LABORATORY STANDARDS, 1998.
10. Federal Express Mail Regulations; Federal Express Co., 1988.
11. *Federal Register*, Vol.57, No.40; February 25, 1992.
12. *Medicare Regulations*: 42 CFR 412.100(b).
13. *Medicare Reimbursement Manual Cost Center*, Part 1, Sec. 2302 & 2313.
14. MacQueen JM: *Tissue Typing Reference Manual*. South-Eastern Organ Procurement Foundation; p 11, 1987.
15. OSHA Guidelines: Federal Register/ Vol. 56. No. 235/ December 6, 1991; Rules and Regulations
16. U. S. Postal Service Domestic Mail Manual, Issue 53, January 1, 1998.

Principles of Cell Isolation: Overview of Current Methodologies

Howard M. Gebel and Robert A. Bray

Purpose

The cellular components of peripheral blood, spleen and lymph nodes are extremely heterogeneous, containing granulocytes, lymphocytes, erythrocytes, platelets, and monocytes. Since most serological assays in an HLA laboratory are best performed using one particular cell type, the isolation and purification of these individual cellular components is usually required. The techniques employed to isolate specific cells rely on differences between the intrinsic and/or extrinsic properties of one cell type compared to the others. Intrinsic properties include the size, density and granularity of a cell, while extrinsic properties include features such as adherence, cell-surface marker expression, and phagocytic capability. There are several techniques or combinations of techniques that can be used to isolate each specific cell type. This section is designed to outline the basic concepts which underlie these isolation techniques. The reader is referred to subsequent chapters for more detailed methodologies.

Mononuclear Cell Isolation By Density

By far, cell density is the most straight forward property used for the isolation of specific cell types. The most popular isolation techniques involve the use of a Ficoll-Hypaque (FH) gradient which has a buoyant density (specific gravity) of 1.077 g/ml, identical to the specific gravity of lymphocytes and monocytes. FH is a combination of a high molecular weight sucrose polymer (Ficoll) and an iodinated organic compound (sodium diatroziate; 3,5-bis acetylamino-2,4,6 triiodobenzoic acid). Since granulocytes and erythrocytes have a significantly higher buoyant density than mononuclear cells, when peripheral blood is centrifuged over a gradient of FH, the granulocytes and erythrocytes pass through the FH and pellet at the bottom of the centrifuge tube. In contrast, platelets which have a buoyant density less than monocytes and lymphocytes, tend to remain in the plasma fraction. Mononuclear cells are found at the FH-plasma interface. However, a significant number of platelets may also be found in the lymphocyte fraction. To eliminate the contaminating platelets, differences in buoyant density between lymphocytes and platelets are again used. Briefly, extended centrifugation of the mixed preparation of cells under a low gravitational force (g) or short-term centrifugation under a high g will preferentially pellet lymphocytes. Although mononuclear cells obtained from FH gradients are, by far, the most common starting material used in an HLA laboratory, there are several situations which require purified populations of platelets, granulocytes or monocytes. Approaches for isolation of these cells will be described later.

In addition to FH, Percoll may also be used as a density separation media. Percoll is a solution of colloidal silica particles (15-30 nm in diameter) coated with polyvinylpyrrolidine. When isolating mononuclear cells from healthy subjects, FH and Percoll give comparable results. However, Percoll is more expensive and since it must be adjusted to a specific density and osmolality before use, probably is not the density gradient of choice for routine mononuclear cell separations. Nonetheless, Percoll does have several uses for the isolation of specific mononuclear cell components. For example, NK cells, which have a large cytoplasm-to-nucleus ratio, can easily be separated from small lymphocytes on a Percoll gradient. More importantly though, Percoll can be used to isolate small lymphocytes from leukemic blast cells. This is a most important feature to a histocompatibility laboratory, since leukemic blast cells are often difficult to HLA-type and may give erroneous results in the mixed leukocyte reaction (MLC).

Lymphocyte Isolation

1. Monocyte Depletion

The specific assays to be performed with the mononuclear cells (e.g., mixed lymphocyte culture vs. class I serology) will determine whether further separation is required. For example, in MLC testing, the mononuclear cell preparation is left intact since both lymphocytes and monocytes are required. In contrast, for serological assays, monocytes are generally removed since their phagocytic properties may result in a false positive reaction due to ingestion of vital dye. The phagocytic property of monocytes can be exploited to eliminate these cells from a lymphocyte preparation. For example, monocytes can ingest iron filings and then be removed either by a magnet or by centrifugation through FH, where the monocytes will now pellet to the bottom of the tube due to their increased density. Another property of monocytes that can be exploited is their ability to adhere to plastic or glass surfaces. Since resting lymphocytes do not have adherence or phagocytic properties, these relatively simple techniques can be used to greatly enrich and purify lymphocytes from peripheral blood.

2. Subset Purification

Frequently, lymphocytes must be further separated, i.e., into T cells and B cells. There are several methods that can be used to enrich for these subsets. Currently, the method that is quickly becoming the standard for purifying T cell and B cell subsets is magnetic microsphere isolation (MMI). In this technique, small magnetic beads are coated with monoclonal antibodies directed against differentiation antigens on T cells or B cells. For example, CD19 or CD20 could be used as the B lymphocyte antigen and CD8, CD4 or CD3 could be used as the T cell antigen. Very briefly, the appropriate microspheres are initially incubated with whole blood or washed buffy coat cells. During this time, beads bind to the specific target cell populations via the monoclonal antibody. Following incubation, the beads (plus their attached cells) are isolated with a magnet. These enriched T or B cells can then be used directly in cytotoxicity assays. However, since the beads are still bound to the lymphocytes, additional steps must be taken to enhance visualization of the cells. For example, pre-staining cells with super-vital dyes such as acridine orange or carboxy-fluorescein diacetate will cause all viable cells to exhibit green fluorescence. Counter staining with ethidium bromide or propidium iodide to visualize dead cells now provides a two color fluorescence method to quantify both dead and viable cells. Magnetic beads have several advantages over other current methodologies; 1) ease of use; 2) significant time saving; 3) applicable to many different types of patient samples; (low white counts and high leukemic blast counts, for example); 4) increased quality of typing particularly for class II antigen determination; and 5) ultimate cost savings due to reduced preparation time and reduced repeat testing. At the present, MMI can be used for both typing and cytotoxicity crossmatching.

In contrast to MMI, the newest isolation technique, sheep red blood cell rosetting is most likely the oldest form of T cell purification. T cells express a surface antigen referred to as CD2, a receptor for the LFA-3 molecule which just happens to be expressed at a high density on sheep erythrocytes. The binding of sheep erythrocytes (usually > 3 RBCs) to a T lymphocyte produces a rosette and a corresponding change in the buoyant density of the T cell such that T cell-RBC rosettes will pass through a FH gradient. The sheep erythrocytes can then be eliminated by hypotonic lysis. A byproduct of this technique is that the non-rosetted (CD2 negative) cells at the FH interface are enriched for B lymphocytes. Thus, the rosette method is a simple and inexpensive technique to enrich for both B and T lymphocytes.

A third technique that also takes advantage of differences in cell surface markers is "panning." This technique depends on the binding of monoclonal or polyclonal antibodies to specific cell surface molecules. Specific cells can be positively or negatively selected using direct or indirect panning, respectively. In direct panning, for example, a mouse anti-human antibody against a particular cell surface marker (such as CD3, a pan-T cell reagent) is incubated with lymphocytes and the cells are then plated on Petri dishes that have been pre-coated with goat anti-mouse-immunoglobulin. In this situation, the antibody-sensitized lymphocytes will bind to the anti-immunoglobulin-coated plates. Non-adherent cells are removed by gently washing and aspiration, while adherent cells are recovered by vigorous washing and agitation of the plate. In the above example, the non-adherent population is enriched for B cells (indirect isolation), while the adherent cells are enriched for T lymphocytes (direct isolation).

A slight modification of this panning technique is the direct isolation of B lymphocytes on Petri dishes coated with anti-human immunoglobulin that is specific for the Fab portion of the molecule; only cells expressing surface immunoglobulin (B cells) will bind to these plates. Non-adherent cells can be discarded or used as a T-enriched population followed by recovery of the adherent B cell-enriched population.

Separation of T and B cells can also be achieved based on their differential adherence to nylon wool: most T cells are nylon wool non-adherent, while B cells are nylon wool adherent. Incubating mononuclear cells on a nylon wool column followed by washing of the column can produce enriched populations of T lymphocytes. Agitation and temperature change (i.e., cold media) will dislodge the B cells from the nylon column and produce an enriched population of B lymphocytes. Residual monocytes adhere to the nylon with a high affinity and are not usually (or easily) dislodged from the nylon.

There are several additional methodologies to isolate T and B lymphocytes, including cell sorting on a flow cytometer and antibody plus complement depletion of specific cell subsets. However while these techniques are quite reliable, they can be quite expensive and their application may only occur in rare circumstances.

In this section, we have described the concepts behind several procedures used to isolate T and B cells from an unfractionated mononuclear cell population. These cells may be obtained from peripheral blood, lymph node or spleen. The choice of a particular technique is quite subjective and highly dependent on the desired end product and the resources available to the laboratory. Furthermore, the type and/or quality of the specimen (peripheral blood from a patient on a respirator, 48-hr old cadaveric spleen, etc.) will dictate which technique(s) is most applicable. It would not be inappropriate to have several isolation techniques practiced. Hence, while all of the described procedures can be quite useful, no one method is necessarily superior to the others.

Monocyte Isolation

Antibodies reactive with vascular endothelial cells may be involved in solid organ allograft rejection. Since some of these antibodies may also react with monocytes, some laboratories may wish to perform monocyte crossmatches. In addition to targets for serological assays, monocytes may also be used in several cellular assays including stimulator cells in mixed lymphocyte cultures, feeder cells for T-lymphocyte cloning, and effector cells for monocyte-mediated cytotoxicity assays. To perform these tests, monocytes must be isolated from other contaminating cell types.

In the section on lymphocyte isolation, we described that adherence could be used to deplete mononuclear cell preparations of monocytes. Similarly, the adherent properties of monocytes may also be used for their enrichment. Thus, mononuclear cells can be incubated on a Petri dish, and the non-adherent cells can be discarded. The adherent cells,

now enriched for monocytes, can be retrieved by vigorous pipetting and/or scraping with a rubber policeman. A recent modification of this adherence technique uses Petri dishes that have been pre-coated with gelatin. In this situation, the monocytes adhere to the gelatin and not directly to the plastic. The non-adherent cells are aspirated, and the Petri dish is then incubated at 37° C. During this time, the monocytes hydrolyze the gelatin and can be easily washed from the plate. This technique is less traumatic than scraping the cells from the plastic. The purity of the monocytes isolated in this fashion is > 95%.

As an alternative to adherence, monocytes may also be enriched based on their density. Such techniques can employ gradients of Percoll-like materials. However, even though isolation of monocytes by density is less time consuming than adherence techniques, the former techniques tend to produce a lower yield, decreased purity and in addition, may result in monocyte activation due to the phagocytosis of, or stimulation by, the separating media.

Granulocyte Isolation

Granulocyte-specific antibodies may be important in patients receiving multiple blood transfusions, since they may be involved in febrile transfusion reactions. Thus, screening for granulocyte-specific antibodies may be applicable and is best performed using purified granulocytes. The most widely employed technique to isolate these cells, again, is based on their cell density, since granulocytes have a higher specific gravity than mononuclear cells. In general, peripheral blood is incubated with dextran to induce sedimentation of the erythrocytes (rouleaux formation). The non-sedimented white cells are then layered over FH, Percoll, or Percoll-like materials (with a specific gravity adjusted to 1.077). Under these conditions, mononuclear cells will band at the interface, and the granulocytes will sediment to the bottom of the tube. The sedimented granulocytes can then be collected, and residual red cells can be eliminated by hypotonic lysis.

Granulocyte Isolation

Platelets are used in a histocompatibility laboratory primarily for absorption of HLA class I specificities from class II containing serum, since platelets do not express class II molecules. Platelets can be isolated based on their low density compared to erythrocytes and leukocytes. Thus, centrifugation of anticoagulated whole blood at a moderate g value results in sedimentation of the white cells and red cells, leaving a platelet-rich plasma (PRP) fraction. The PRP can be further enriched for platelets by subsequent centrifugation following dilution with appropriate buffers. This simple technique can be repeated until the platelets are essentially devoid of any other cellular material.

Summary

This section has outlined some of the various characteristics that can be employed to isolate or enrich differing cell types. As stated, the techniques that are eventually used in the laboratory depend on several factors such as laboratory resources and the characteristics of the starting materials received. We would like to stress the point that regardless of the techniques utilized for cell isolation, the actual purity of the cell preparation should be verified. Thus, even though you may have followed manufacturers directions explicitly for the isolation of B lymphocytes from peripheral blood, without good documentation of the accuracy of the technique, use of such cells could be disastrous. This holds true for documenting the presence of the other cellular constituents as well. Although documenting the purity of the final cell preparation may take a small amount of additional time, in our estimation, it is well worth the effort.

The detailed procedures for each of the isolation methods outlined in this chapter can be found in subsequent chapters of this manual. Do not hesitate in using them and referring to them often. Remember when all else fails – READ THE DIRECTIONS.

References

GENERAL

1. Cerilli J, Brasile L, Clarke J and Galouzis T, The vascular endothelial cell-specific antigen system. Three years experience in monocyte crossmatching. Transplant Proc 17:567, 1985.
2. Cline MJ and Lehrer RI, Phagocytosis by human monocytes. Blood 32:423, 1968.
3. Evans RL, Faldetta TJ, Humphreys RE, Pratt DM, Yunis EJ and Schlossman SF, Peripheral human T cells sensitized in mixed leukocyte culture synthesize and express Ia-like antigens. J Exp Med 148:1440, 1978.
4. Forbes, JF, and Morris PJ: The use of lymph node and spleen lymphocytes for HLA typing of cadaver kidney donors. Transplantation 13:444, 1972.
5. McCullough J, Weiblin BJ, Clay ME and Forstrom L, Effect of leukocyte antibodies on the fate in vivo of Indium-III-labeled granulocytes. Blood 8:164, 1981.
6. Schiffer CA, Aisner J, Daly PA, Schimpff SC amd Wiernik PH, Alloimmunization following prophylactic granulocyte transfusion. Blood 54:766, 1979.
7. Vassalli P, Jeannet M, and de Moerloose P et al, A screening program for anti-DR typing reagents. Tissue Antigens 13:77, 1979.

ISOLATION PROCEDURES

8. Boyum A, Isolation of leukocytes from human blood. Further observations methyl, cellulose, dextran, and ficoll as erythrocyte aggregating agents. Scand J Clin Invest 97 (Suppl):31, 1968.
9. Boyum A, Separation of lymphocytes, lymphocyte subgroups and monocytes: A review. Lymphology 10:71, 1977.

10. El-Awar N, Terasaki PI, Perdue S, Cicciarelli J and Mickey MR, Discrimination of T, B and null lymphocytes by electronic sizing. Tissue Antigens 15:346, 1980.
11. Gandernack G, Leivestad T, Ugelsted J, and Thorsby E, Isolation of pure functionally active CD8+ cells. Positive selection with monoclonal antibodies directly conjugated to monosized microspheres. J Immuno Meth 90:179, 1986.
12. Grier JO, Abelson LA, Mann DL, Amos DB, and Johnson AH, Enrichment of B lymphocytes using goat anti-human F(ab)2. Tissue Antigens 10:236, 1977.
13. Freundlich B and Audalovic N, Use of gelatin/plasma coated flasks for isolating human peripheral blood monocytes. J Immunol Meth 62:31, 1983.
14. Gutierrez C, Bernabe RR, Vega J and Kreisler M, Purification of human T and B cells by a discontinuous density gradient of percoll. J Immunol Meth 29:57, 1979.
15. Hansen T and Hannestad K, Direct HLA typing by rosetting with immunomagnetic beads coated with specific antibodies. J Immunogenet 16:137, 1989.
16. Jondal M, Holm G and Wigzell H, Surface markers on human T and B lymphocytes. I. A large population of lymphocytes forming nonimmune rosettes with sheep red blood cells. J Exp Med 136:207, 1972.
17. Lea T, Smeland E, Funderud S, Vartdal F, Davies C, Beiske K and Ugelstad J, Characterization of human mononuclear cells after positive selection with immunomagnetic particles. Scand J Immunol 23:509, 1986.
18. Mage MG, McHugh LL and Rothstein TL, Mouse lymphocytes with and without surface Ig: preparation scale separation in polystyrene tissue culture dishes coated with specifically purified anti-immunoglobulin. J Immunol Methods 15:47, 1977.
19. Muller-Eckhardt G, Kolzow S, Conrath K, and Hofman O, HLA typing and lymphocyte crossmatches using conventional isolation and immunobeads. Vox Sang 61:99, 1991.
20. Pertaft H, Laurent TC, Lass T, et al, Density gradients prepared from colloidal silica particles coated by polyvinylprolidone (Percoll). Anal Biochem 88:271, 1978.
21. Timonen T and Saksela E, Isolation of human natural killer cells by density gradient centrifugation. J Immunol Methods 36:285, 1980.
22. Vartdal F, Bratlie A, Gaudermack G, Funderud S, Lea T, and Thorsby E, Microcytotoxicity HLA typing of cells directly isolated from blood by means of antibody-coated microspheres. Transpl Proc 19:655, 1987.
23. Worlock AJ, Sidgwick A, Horsburgh T and Bell P, The use of paramagnetic beads for the detection of major histocompatibility complex class I and class II antigens. Biotechniques 10:310, 1991.
24. Wysocki LJ and Sato VL, "Panning" for lymphocytes: a method for self selection. Proc Natl Acad Sci 75:2844, 1978.

Density Gradient Isolation of Peripheral Blood Lymphocytes

Brenda B. Nisperos

Purpose

Since isolation of lymphocytes from peripheral blood must be simple and rapid, the technique chosen must not compromise the essential requirements of purity and viability of the cell suspension. The most widely used method is density gradient centrifugation, whose principle is based on the centrifugal force, density, and viscosity of the separation medium. The most commonly used gradients are discussed below.

Ficoll-hypaque (FH) separation. Ficoll is a high molecular weight sucrose polymer, which contributes viscosity and promotes molecular formation of red cells. Hypaque is an iodinated organic compound which increases density of the mixture. When these solutions are combined, the density is adjusted to 1.077, which is denser than lymphocytes, platelets, and monocytes, but less dense than granulocytes and red cells. This density difference in blood cells is the basis for an efficient separation method, and under appropriate centrifugation conditions of force and time, red cells,granulocytes and some monocytes will sediment through the medium, while lymphocytes and residual platelets will remain in the plasma-Ficoll-Hypaque interface. Diluted whole blood can then be layered directly on the gradient, or the gradient may be placed under the diluted blood. Buffy coat enriched plasma is commonly used to reduce the red cell contamination.

Percoll separation. Percoll separation can be performed with fluid, which consists of polyvinylpyrrolidone-coated silica particles. Using different concentrations, it can be used to isolate lymphocytes, monocytes, platelets, granulocytes and dead cells. It is primarily used for separation of the lymphoid cell population. Although Percoll is used in a fashion similar to that of Ficoll-Hypaque gradient, there is a difference in principle and methodology. Instead of layering over gradient, cells are mixed with the most dense layer. Centrifugation forces the lighter cells through the layers until the cells reach their density level. In most HLA laboratories, the use of Percoll is more a purification or troubleshooting procedure than a first-step isolation technique, as was developed in the University of California, Los Angeles (UCLA) Histocompatibility Laboratory.

Preloaded Commercial Separation Tubes

Leucoprep, distributed by Becton-Dickinson Immunocytometry Systems, is an uncoated tube containing lymphocyte separation medium that is made up of two immiscible layers of fluid. One is a denser solution of polysaccharide and sodium diatriuzoate while the other is a polyester gel, which prevents mixing of blood with polysaccharide solution. The principle of separation is similar to that of FH in that blood, diluted or undiluted, or buffy coat is layered on top of the media and centrifuged. Lymphocyte separation using these tubes seems to be a little faster and easier compared to the standard FH technique. Leucoprep tubes are available in 13 x 100 mm tubes that can separate up to 5 ml of blood and in 16 x 125 mm tubes, which can separate up to 10 ml of blood.

Reagents

Ficoll-Hypaque (FH) Solution

Distilled water	150 ml
Ficoll powder	9 g
75% Hypaque	20 ml

Add Ficoll to distilled water and mix until completely dissolved. Add the Hypaque and additional distilled water until refractive index = 1.353 or until the specific gravity is = 1.077. For sterile preparation, filter with 0.45 or 0.2 m filter.

Percoll

Percoll (Percoll-X), a stock solution developed by the UCLA Histocompatibility Laboratory, is one part of 10X PBS and 9 parts of Percoll. The stock solution is used to prepare the different concentrations of 40%, 55% and 65% of Percoll. With 1X PBS, the stock solution is stable at 4° C for a long period of time.

Hank's balanced salt solution (HBSS)
RPMI + 5% fetal calf serum (FCS)
McCoy's media + 15% FCS

Procedures

Ficoll-Hypaque (FH)

Procedure A: Use for isolation of peripheral blood lymphocytes from 10-15 ml of anticoagulated blood and utilizing buffy coat layer.

1. Centrifuge 15 ml heparinized blood for 10 min at 700-900 g to obtain buffy coat of leukocytes. This can also be obtained by use of aggregating agent (5% dextran or 1% methyl cellulose) as follows: mix 1 ml of the aggregating agent to 5 ml of whole blood and allow red cells to sediment at 37° C for 15 min.
2. Hold the tip of a Pasteur pipette slightly above the buffy coat and, with a swirling motion, aspirate approximately 2 ml of the buffy coat layer including plasma.
3. Transfer the aspirate to a clean, capped 16 x 100 mm tube containing approximately 5 ml HBSS (1X) and mix well.
4. Dispense 4 ml of FH gradient solution into 16 x 100 mm test tube. It is important to warm the FH at 22° C (RT) prior to use. The ratio of dilute blood to FH should not exceed 3:1.
5. Carefully layer the buffy coat suspension over the FH and centrifuge for 20 min at 1000 g at 22-25° C. Note that at higher temperatures, red cell aggregation is increased while lymphocyte viability is decreased. At lower temperature the time of separation is increased. After centrifugation, the mononuclear cells can be found as a narrow band at the interface between the plasma/diluent and separation fluid.
6. With a Pasteur pipette, aspirate all of the mononuclear cell layer, which will be located mostly around the periphery of the tube. Thus, it is necessary to move the pipette over the whole cross-sectional area of the tube.
7. Transfer to a 16 x 100 mm tube. Dilute with HBSS.
8. Centrifuge at 600 g for 5-10 min. Remove supernatant and repeat washes twice.
9. Resuspend cell pellet in 1-2 ml culture medium with 5% FCS for viability, purity and cell concentration testing.

Procedure B: Use when processing large amounts of blood.

1. Distribute heparinized blood into an appropriate number of 50 ml Falcon tubes, 15-20 ml of whole blood per 50 ml Falcon tube.
2. Dilute the whole blood with equal volume of Hank's (HBSS) or cell culture medium. Cap the tube and mix carefully by inverting the tube several times before proceeding to step #3.
3. Underlayer the diluted whole blood with 10-12 ml FH gradient using a large bore 10 ml pipette and an automatic pipettor making sure that the interface between the blood and FH is as distinct as possible. This can be accomplished by adding the FH slowly and carefully to the bottom of the Falcon tube. Avoid introducing bubbles into the tube as this will disturb and obscure the interface layer between the FH and diluted blood.
4. Centrifuge at 700-900 g for 20 min.
5. Aspirate all of the mononuclear (lymphocytes, monocytes and platelets) cell layer at the interface above the density gradient, including some of the plasma but as little of the Ficoll as possible using another large bore 10 ml pipette and automatic or manual pipettor. Transfer to another 50 ml Falcon tube if more than 20 ml of whole blood is being isolated or to a 15 ml tube if less than 20 ml of blood is being isolated.
6. Centrifuge at 700-900 g for 5 min (first wash). Aspirate or decant supernatant. Combine cell pellets and resuspend in HBSS.
7. Centrifuge at 400-500 g for 6 min (second wash). Aspirate or decant supernatant and resuspend cell pellet in HBSS.
8. Centrifuge at 400-500 g for 6 min for final wash. Aspirate or decant supernatant.
9. Resuspend cell pellet in 5-10 ml of complete media with FCS (i.e., RPMI + 20% FCS or McCoys + 15% FCS).
10. The cell suspension is now ready for counting, viability, purity and cell concentration testing.

Percoll

1. Resuspend cell pellet (from step 9, FH Procedure) in 1 ml of 65% Percoll in a 12 x 75 mm plastic tube.
2. Over the 65% Percoll, layer the following in the order given: 1 ml of 55% Percoll, 1 ml of 40% Percoll, and 1 ml of medium. Centrifuge at 1000 x g for 10 min.
3. Remove the first two of the three interfaces, the top (40%) of which must contain the platelets and the dead cells, and the middle (55%) of which must contain monocytes. The bottom interface, just above the 65% Percoll layer, must contain the lymphocytes.
4. Aspirate the lymphocyte layer, add medium and wash cells to remove Percoll. (See steps 7 and 8 above).
5. Resuspend in culture medium with 5%HIFCS, adjusting cell concentration for use.

A stock solution (Percoll-X) of 1 part 10X PBS and 9 parts of Percoll is used to prepare the different density solution using 1X PBS as diluent. The stock solution is stable at 4° C for a long period of time.

Troubleshooting

	PROBLEM	POSSIBLE CAUSE	SOLUTION
1.	Lymphocyte interface is indistinctive or thin	Hyperlipemic blood sample	Use 8-hr fasting blood sample
		Centrifugation force is too low and time too short	Adjust centrifugation to 2000 x g for 25 min
		Mixing of blood and FH	Observe care in layering
2.	Presence of RBC in the lymphocyte band	Density of RBC altered due to disease	Resuspend cell button and centrifuge at 1000 x g for 3-4 sec. RBC will settle to bottom, lymphocytes in supernatant
		Excessive harsh mixing of blood	Hypotonic lysis: treat cells with Tris-buffered NH_4Cl
3.	Low lymphocyte yield	Low WBC of blood donor	Repeat when WBC increases or obtain a larger blood sample
		Buffy coat left on RBC ¼ inch of RBC layer	Collect buffy coat to extend to ¼ inch of RBC layer
		Cells left in interface	Careful collection
		Cells clumping	Resuspend in medium and agitate gently to release lymphocytes
		Pellet not complete	See #4
4.	Mononuclear cells (MNC) will not pellet after washing	Insufficient volume of wash medium	Increase volume of wash medium
		Incomplete mixing of MNC band with wash medium	Resuspend cells, mix well and centrifuge again
		Collection of Ficoll layer exceeds 2.5 ml	Extra wash with increased volume of medium
5.	Cell viability <90%	Blood samples >24 hrs old	Use fresher sample
		Lack of protein in wash medium	Use 0.1% Cohn Fraction V BSA with PBS or use culture medium with FCS for washing
6.	>3% granulocyte contamination	Density of granulocytes altered due to disease state or abnormal blood sample	Resuspend cell button in 1 ml of 40% Percoll, spin at 2000 x g for 1 min. Resuspend cells in medium and wash twice
		Specific gravity of FH is too high	Check specific gravity of FH. Must be 1.077. Perform differential centrifugation and other purification technique such as thrombin, use of carbonyl iron, and Lympho-Kwik™ reagent
7.	Platelet contamination	Blood sample >24 hrs old	If possible, use fresher sample
		Blood drawn in heparin	Use defibrinated blood in ACD
			Use purification technique such as thrombin, ADP and percoll methods

References

1. Boyum A: Separation techniques for mononuclear blood cells. HLA Typing: Methodology and Clinical Aspects Vol. I: p 2, 1976.
2. Mittal KK, Fotino M and Menon AK: Isolation and Purification of Peripheral Blood. In: *AACHT Laboratory Manual.* Zachary AA and Braun WE, ed. Am. Assoc. for Clinical Histocompatibility Testing. NY, I-2-1, 1981.
3. Garcia ZC and Gal K: Cell preparation. In: *Tissue Typing Reference Manual.* MacQueen JM, ed; South-Eastern Procurement Foundation Richmond, p 11.1, 1987.
4. Miller WV and Rodey G: *HLA Without Tears.* American Association of Clinical Pathology, Chicago, IL, 1981.
5. Ray JH: *NIAID Manual of Tissue Typing Techniques,* 1979-1980. Ray JH, Bethesda, Maryland, 1979.
6. HLA Lab Procedures Manual. III-1. Isolation of Lymphocytes from Whole Blood, Clinical Immunogenetics Laboratory, Fred Hutchinson Cancer Research Center, Seattle, WA, p 2, 1990.
7. Tips for Techs, *ASHI Quarterly,* Fall 1984.

PRODUCT LITERATURE

1. Lympho-Kwik™ by One Lambda Inc., 2/16/95

Density Gradient Isolation of Peripheral Blood Lymphocytes: Augmentation with Monoclonal Antibodies (Lympho-Kwik™)

Brenda B. Nisperos

Purpose

Additional techniques for isolation of lymphocytes for histocompatibility testing have been developed utilizing other properties of leukocytes such as surface charge, surface immunoglobulins and specific recognition sites. The University of California, Los Angeles (UCLA) Tissue Typing Laboratory has developed Lympho-Kwik™ isolation medium (a cocktail of monoclonal antibodies) to separate lymphocytes from non-lymphocytic cells by lysing the non-lymphocytic cells with specific monoclonal antibodies and complement. The lysed cells are then separated from the lymphocytes by density centrifugationn. Currently, there are five different reagents used for each specific lymphocyte separation that are now available through One Lambda, Inc. These reagents and their uses are listed in Table 1.

Table 1: Lympho-Kwik™ Lymphocyte Isolation Reagents

Reagent	Cells Isolated	Cells Eliminated						Applications
		RBC	G	P	M	T	B	
Mononuclear Cell (MN)-KWIK	Lymphocytes monocytes	+	+	+				MLC; HLA class I typing; T/B separation
T/B Cell (T/B)-KWIK	T and B Lymphocytes	+	+	+	+			T/B separation; T/B ratio; HLA class I typing
T -KWIK	T lymphocytes	+	+	+	+		+	Cleaner class I typing
B Cell (B)-KWIK	B lymphocytes	+	+	+	+	+		HLA-DR, -DQ and DP typing
T Helper Cell (TH)-KWIK	T helper Lymphocytes	+	+	+	+		+	Functional studies
G: granulocytes; P: platelets; M: monocytes; T: T lymphocytes; B: B lymphocytes; TH:T-Helper cells								

Recommended Specimen

Blood obtained in heparin or acid citrate dextrose

Unacceptable Specimen

Clotted blood
Specimen older than 2 days

Reagents

1. Lympho-Kwik™ kits (depending on desired cell separation)
2. Phosphate-buffered saline (PBS)
3. Hank's or McCoys' medium

Instrumentation

1. Centrifuge
2. 37° C waterbath or heat block

Procedures

T, T/B, T-Helper(TH) and MN Lympho-Kwik™

Description: Lympho-Kwik™ is a premixed cocktail of monoclonal antibodies, complement and a stable density gradient developed for isolation of specific lymphocyte populations. The method assures maximum cell yield and purity.

Preparation and Storage:

1. Thaw Lympho-Kwik™ in cold tap water. Use immediately.
2. Keep reagent immersed in ice after thawing to insure maximum reagent activity.
3. Store Lympho-Kwik™ at -65° C or below upon receipt.
4. Lympho-Kwik™ may be filtered through a 0.2 micron filter.
5. Do not thaw Lympho-Kwik™ more than twice.

DIRECTION FOR USE

A. Isolation of small numbers of lymphocytes (up to 3 x 10^6 lymphocytes)
   1. Centrifuge 5-15 ml of whole blood (citrated or heparinized) at 400 – 900 g for 10 min.
   2. Wash 0.1 ml of buffy coat in 1 ml 1X PBS and centrifuge at 1000 g for 1 min. Discard supernatant completely. It is important to start with no more than 0.1 ml of buffy coat, otherwise overloading may occur.
   3. Add 0.8 ml of Lympho-Kwik™ or entire contents of plastic pipet, mix well, and incubate at 37° C in a waterbath or heat block. Occasionally mix by inverting capped tube. Incubate according to the following schedule:
      a. T cell: 20 min
      b. T/B cell: 30 min
      c. MN cell: 15 min
   4. Mix well with a pipet to break up clumps, layer 0.2 ml of medium (PBS, Hank's or McCoy's) over cell preparation and centrifuge at 2,000 g for 2 min. Remove and discard floating cell layer and supernatant.
   5. Wash lymphocyte pellet twice with PBS, and then centrifuge at 1000 g for 1 minute. Resuspend in McCoy's medium and adjust to working concentration.

B. High Yield Isolation Procedure (greater than 3 x 10^6 lymphocytes)
   When large numbers of lymphocytes are desired, this procedure can be used for T cell and mononuclear isolations.
   1. Centrifuge 10-20 ml of citrated or heparinized whole blood for 10 min at 400-900 g
   2. Transfer the entire buffy coat from step A1 above to 1 ml Fisher tube and spin for 1.5 min at 1500 g.
   3. Remove buffy coat and distribute it equally among three Fisher tubes containing PBS.
   4. Centrifuge at 1000 g for 1 min, discard supernatants, and resuspend each pellet in 0.8 ml of Lympho-Kwik™.
   5. Incubate cells at 37° C in waterbath or heatblock for the respective time period.
   6. Mix well with a pipet to break up clumps, layer 0.2 ml of medium (PBS, Hank's or McCoy's) over cell preparation and centrifuge at 2000 g for 2 min. Remove and discard floating cell layer and supernatant.
   7. Wash lymphocyte pellet twice with PBS using a 1000 g spin for 1 min. Resuspend in McCoy's medium and adjust to working concentration.

Troubleshooting

1. Problem: Excessive buffy coat.

   Solution: If greater than 0.1 ml of buffy coat has been drawn, centrifuge lymphocytes in PBS at 2000 g for 2 min, discard supernatant, and transfer only the white layer to a Fisher tube containing 0.8 ml of Lympho-Kwik™. Then continue normal procedures.

2. Problem: Excessive red cells.

   Solution: Cloudy supernatants indicate excessive red cell contamination. This may be due to inadequate incubation time or low incubation temperature. Check both time and temperature and repeat the procedure with another 5-10 min. incubation period.

3. Problem: Clumped red cells or granulocytes during wash.

   Solution: Remove by using a "soft spin" of 1000 g for 3 sec. Transfer supernatant to a clean Fisher tube and continue washing procedures.

It is important to use a heatblock or waterbath for incubation. This maximizes the reactivity of Lympho-Kwik™.

B-Kwik

B-Kwik is a medium that lyses and separates non-B cells from B cells. T cells cannot be recovered by this technique. The following procedure will yield 0.5-2 x 10^6 B lymphocytes.

1. Isolate not more than 10 x 10^6 whole lymphocytes by method of choice, preferably Ficoll-hypaque (FH).
2. Pellet the lymphocytes in a Fisher tube at 1000 g for 1 min. Discard supernatant completely.
3. Add 0.8 ml of Reagent 1 (included in the kit) and mix well.
4. Incubate at 37° C for 60 min in a heatblock or waterbath; occasionally mix by inverting capped tube.
5. Layer 0.2 ml of normal PBS or similar medium on top of Reagent 1.
6. Centrifuge at 2000 g for 2 min.
7. Discard supernatant and add 0.5 ml of Reagent 2 (included in the kit). Mix well.
8. Centrifuge at 2000 g for 2 min.
9. Discard supernatant and wash lymphocytes with normal PBS, then centrifuge at 1000 g for 1 min. Repeat twice.
10. Resuspend in McCoy's medium and adjust to working concentration.

Troubleshooting

1. Problem: Excessive background; B cell yield is greater than 20% of whole lymphocyte yield.

   Solution:
   a. Samples should not be older than two days.
   b. The initial whole lymphocyte preparation should be clean. Excessive contamination by red cells and granulocytes weakens B cell isolation reagent activity.
   c. Incubate at 37° C. Higher temperatures cause damage to the B cells.
   d. Use not more than 10 x 10^6 whole lymphocytes. More cells overload the reagent. Corrective procedure: repeat dosage of Reagent 1 and 2.

2. Problem: Red cell contamination

   Solution: If excessive after FH separation, we recommend eliminating the red cells by either lysing with ammonium chloride solution or agglutination with appropriate anti-red cell antibody. Smaller amounts of red cells should lyse during initial contact with Reagent 1.

References

PRODUCT LITERATURE

1. Lympho-Kwik™ by One Lambda, Inc., 2/16/95

Isolation of Lymphocytes from Lymph Nodes and Spleen

William M. LeFor

Purpose

Typing and crossmatching with lymphocytes obtained from lymph nodes or spleen is usually required for shared cadaver organs. Importantly, this cell source may also be that of choice for many local cadaver donors, the majority of which have been treated with steroids. Under these circumstances the preparation of lymphocytes from peripheral blood is difficult, time consuming, and may result in a less than adequate population of target cells. Logistical problems, sample shipping conditions, and massive transfusion of donors add to the difficulties of using peripheral blood as the lymphocyte source in many cases.

Isolation of target cells from nodes and spleen is rapid, quite simple, and provides large numbers of cells of known donor origin with excellent viability, low background at time of test reading, and minimum contamination with debris or unwanted cells. In fact, those who have worked with such cells, particularly those from lymph nodes, usually express the wish that this cell source could be used for all activities. Under normal circumstances, preparation of cells from one or two small nodes or a piece of spleen provides sufficient cells for complete donor typing, preliminary crossmatch screening, and final crossmatching with many patients. Thus, the need to stop and prepare more target cells midway through the testing process is obviated. In addition, leftover nodes or spleen fragments are an extremely valuable resource for the laboratory. Large numbers of typed cells are easily retrieved and can be stored frozen as part of a library of cells for future use in a variety of ways. If desired, cells from nodes and spleen can be prepared under sterile conditions with minimum extra effort.

Although some laboratories are successful in employing pre-harvest peripheral blood samples, many wait until nodes and spleen are retrieved at the time of organ procurement. We feel this causes unnecessary prolonged ischemia time and is particularly troublesome when organs are to be shared. Additionally, earlier knowledge of donor parameters facilitates multiple organ procurement from the same donor. Since 1982 we have employed a protocol which we simply term “pre-typing.” Following signed permission specifically for the procedure, inguinal lymph nodes are excised at the bedside (a reimbursed expense for the organ procurement organization) and transported to the laboratory. The testing time required is dependent upon a variety of factors including condition of the specimen, number of patients to be screened, test methods employed, etc. Our experience with 548 local cadaver donors over a 5-year period is as follows. After receipt of the nodes and a small blood sample, we have completed red cell typing, infectious disease serologies, HLA typing, and preliminary crossmatch screening of patients by 4 hrs. Data is entered in the UNOS computer at that time. By 6½ hrs offers for sharing organs have been made and the clinicians have notified us which local patients are to undergo final crossmatching.

These final crossmatches are generally completed by 12 hrs. The mean time of organ procurement with these particular donors was 9.2 hrs (medium = 8.4 hrs) after we received the nodes. This “pre-typing” protocol with lymph node cells has been extremely useful and beneficial. When offers to share organs were made it was completed before procurement with 73% of the donors. Additionally, our ischemia times are short since 68% of the kidneys were procured within 6 hrs of our having completed final crossmatching. Potential heart and liver transplant recipients are crossmatched simultaneously with donor typing and the results reported to the clinicians well in advance of procurement with virtually every local donor.

The lymphocyte isolation procedure described below has evolved over the course of time in our laboratory. The intent has been to prepare optimal target cells in the shortest possible period of time using the gentlest and mildest conditions.

Specimen

Media containing excised lymph nodes and/or spleen tissue that are labeled according to ASHI standards.

Reagents and Supplies

Reagents

1. Lympho-Kwik-MN™, 0.75 ml pkg. as provided, stored at -80° C
2. Deoxyribonuclease (DNase) enzyme at 2.75 mg/ml, 100 µl aliquots in Beckman tubes, stored at -80° C
3. Ficoll-Hypaque (FH)
4. RPMI 1640 medium supplemented with N-2-Hydroxyethylpiperazine-N’-2-Ethane Sulfonic Acid (HEPES) and Penicillin-Streptomycin and 5% FCS
5. Red-Out (human red cell agglutination reagent)

Supplies

1. Plastic backed absorbent pad
2. Gloves (preferably rubber)
3. Glasses (eye protectors)
4. Petri dishes (15 x 60 mm or 20 x 100 mm)
5. Syringe (1 or 5 ml)
6. Hypodermic needles (#26 or #23 gauge)
7. Gauze pads (2" x 2" or 4" x 4")
8. Test tubes (16 x 100 mm)
9. Adson tissue forceps, 4¾" (see your surgical colleagues)
10. Iris scissors, 4¼", straight (see your surgical colleagues)

Instrumentation/Special Equipment

1. Water bath
2. Centrifuge and rotor capable of attaining appropriate speeds and holding specified tubes
3. Biological Containment Hood if needing to insure sterility of specimen

Calibration

Centrifuge and rotor should be calibrated to generate appropriate g forces. All thermometers need to be calibrated to one certified by the National Bureau of Standards (NBS). Hoods need to have air flows calibrated to produce desired protective effect.

Quality Control

Standard reagent and equipment QC should be performed and must be documented.

Procedures

Isolation of Lymphocytes From Lymph Nodes

In preparation of working with either lymph nodes or spleen, place reagents and materials on absorbent pad. If sterile cells are required use sterilized materials, aseptic techniques, and perform the isolation in an appropriate hood. Glove, whether or not cells are collected sterilely. Because of the possibility of splashing, eye protectors should be worn.

1. Transfer the node-containing fatty material to a Petri dish containing sufficient medium to keep the tissue moist. If the nodes are not visible they can be "felt" within the fatty tissue by transferring it to a gauze pad with tissue forceps and applying gentle pressure with the edge of the scissors.
2. Gently hold an edge of the node with forceps, and trim away fat and connective tissue and particularly any blood vessels, with the scissors. This is most easily accomplished on the gauze pad with frequent dipping of the node into media to rinse it and keep it moist. Avoid cutting the node. Trimmed fat and connective tissues are wiped off the scissors onto the gauze pad. Rinse the nodes in medium to free them of any fat globules.
3. Transfer the cleaned nodes to a new Petri dish containing just enough medium to keep them moist. Gently hold the node with the forceps and puncture it in 4-5 sites with a needle.
4. Fill the syringe with fresh medium and slowly inject the medium into the node. The medium escaping from the node is turbid with lymphocytes. Continue the process with fresh medium until sufficient cells are obtained or until the node has been depleted of cells, in which case it will float.
5. Transfer the cell suspension to 16 x 100 mm tubes and centrifuge at about 1000-1200 x g for 4 min.
6. Add 100 µl DNase to the vial of Lympho-Kwik-MN™, resuspend the cell pellet in this mixture, and incubate in a 37° C water bath for 15 min.
7. Gently mix the cell suspension and overlay with 1 ml of medium to achieve 2 phases. Centrifuge at 1000-1500 x g for 1-2 min.
8. Remove and discard the supernatant. Resuspend and wash the cell pellet twice at 1000-1200 x g for 4 min.
9. Resuspend and count the cells, check their purity and viability, and adjust to the desired concentration.

Isolation of Lymphocytes From Spleen

Lymphocytes from spleen tissue can be recovered by the same technique used with lymph nodes. Because of the larger number and proportion of contaminating cells (macrophages, platelets, erythrocytes, etc.) however, it is convenient to start with a larger number of cells and use FH as an initial preparation step. Using the same reagents and materials described above, proceed as follows.

1. Trim a 2-3 cm^3 wedge of spleen free of the larger pieces of connective tissue and blood vessels, and rinse.
2. Transfer the wedge to a new Petri dish, containing media as in step #3 above, and gently squeeze with forceps to liberate cells. Divide the cell suspension obtained into 2-6 16 x 100 mm tubes, diluting with media until it looks like "fruit punch."

3. Add one drop of "Red Out" to each tube and mix. Allow to sit at room temperature for 5 min. prior to centrifugation. You may underlay with FH during this time.
4. Underlay each of the four tubes with 3 ml FH, and centrifuge at about 1200 x g for 15 min.
5. Transfer the cells from the white band to two 16 x 100 mm tubes. Fill the tubes with medium and centrifuge at 1000-1200 x g for 4 min.
6. Add 100 µl DNase to the vial of Lympho-Kwik-MN™, transfer the mixture to one of the tubes and resuspend the cell pellet. Repeat for all tubes. Incubate in a 37° C water bath for 15 min.
7. Proceed with steps #7, 8, and 9, as described above, for each tube.

Results and Interpretation

One empirically gets the feeling that very gentle handling of lymphocytes with a minimum number of steps during the purification process results in a much more satisfactory target cell population. It also seems reasonable to reduce the bulk of contaminants (e.g., trim the node, use the FH step with spleen cells) prior to Lympho-Kwik-MN™ treatment so as not to overload the reagent's capacity to purge unwanted cells. We have found that combining the DNase and Lympho-Kwik-MN™ treatments into one procedure provides excellent results, saves time, and reduces the number of times the cells are subjected to changes in temperature (room temperature to 37° C to room temperature), and centrifugation.

The procedure described above employs Lympho-Kwik-MN™ which was selected because of the shorter incubation time required and the fact that one preparative procedure provides cells which are used for both conventional HLA-A,B,C typing and HLA-DR typing by the 1-color technique. Thus, although the resulting preparation contains monocytes in addition to lymphocytes, we have not found them to cause problems in typing.

Additionally, this preparation can be further separated into T cell, B cell and monocyte components for crossmatching purposes. We have used either the nylon wool absorbent or immunomagnetic bead method to our complete satisfaction. There is no reason to think that Lympho-Kwik-T/B™ (to obtain T and B lymphocytes devoid of monocytes), Lympho-Kwik-T™ (to obtain T cells) or Lympho-Kwik-B™ (to obtain B cells) would not yield equally satisfactory results, dependent on the target cell population one wants. We have not tested the combined DNase treatment with the latter three Lympho-Kwiks™. Therefore, such a system should be tested prior to use, or simply do the DNase and Lympho-Kwik™ treatments as two separate procedures.

Depending on anticipated viability and the number of contaminating cells, we find that the 100 µl of DNase plus one vial of Lympho-Kwik -MN™ is sufficient for about 10^7 cells. As one gains experience, cell numbers can be estimated by the volume and turbidity of the cell suspension, or pellet size. Counting cells at the various steps of the procedure is recommended prior to attainment of such experience. Multiples of the reagents can be used to prepare larger numbers of cells. If poor cell viability is anticipated, for example with a shipped spleen, 200 µl of DNase is added to the vial of Lympho-Kwik™.

Calculations

Not Applicable

Procedure Notes

Troubleshooting

The resulting target cell population must be examined for adequacy in terms of numbers of desired cells, their viability, and absence of debris and contaminating cells. If not satisfactory, the DNase, Lympho-Kwik-MN™ or ficoll-hypaque procedures described above can be repeated. Alternatively, one could use procedures such as different Lympho-Kwiks™, carbonyl iron ingestion, and other techniques described elsewhere in this manual. Our experience has been that these further steps are seldom required.

Proper storage and transport of nodes and spleen fragments is important for the recovery of an adequate preparation of target cells. The following protocol is recommended. About 30 ml of sterile HEPES-buffered RPMI-1640 tissue culture medium containing antibiotics and 5% fetal calf serum (FCS) is placed in sterile screw-cap 50 ml plastic tubes. These tubes are periodically prepared, provided to the organ procurement personnel, stored at 4° C, and taken with them for each case. Cutting the spleen into fragments allows better perfusion of the cells, and hence better viability. Nodes or pieces of spleen are placed in these tubes and transported to the laboratory under cool but not cold conditions. This same protocol is suggested when tissue typing materials are shared with another laboratory.

For unknown reasons, B lymphocytes isolated from nodes or spleen appear to be more fragile than those from peripheral blood. Thus, gentleness and care in their isolation and use is necessary, and addition of FCS (5%) to the tissue culture medium is helpful in maintaining viability. This same feature, however, makes them ideal target cells for the screening of complement (see Quality Control of Complement).

Earlier editions of tissue typing manuals (SEOPF 1976, AACHT 1981) suggested that inguinal nodes should be avoided. We do not understand the reason for that recommendation, have had few problems, and have routinely employed inguinal nodes in our "pre-typing" protocol. This also pleases the organ procurement personnel as these nodes are much easier to find than those in the mesentery.

Common Variations

There are probably as many variations in preparing cells from nodes and spleen as there are laboratories. The importance of testing any given system to determine what works best in your hands cannot be stressed enough. In these regards, careful consideration must be given to the target cell populations one wishes to obtain, how they will be employed, methods used for class I and II typing and crossmatching, and time constraints. For example, one may wish to treat one aliquot of cells with Lympho-Kwik-T™ for HLA-A,B,C typing and T cell crossmatching and another with Lympho-Kwik-B™ to prepare B cells for HLA-DR typing and crossmatching. Because of the large number of cells obtained from nodes and spleen, these tissues are ideal for experimenting with different preparative procedures.

Although use of Lympho-Kwik-MN™ and/or DNase may not be required in every case, we have found that their standard application consistently provides target cell populations of excellent viability (i.e., very low background at the time of reading), which are suitable for the typing methods employed. Because of the few contaminants, simple teasing out and washing of lymphocytes from nodes may be sufficient. In contrast, those from spleen tissue are heavily contaminated and preparative steps such as carbonyl iron treatment, use of FH, etc., are required. These and other techniques are described in detail elsewhere in the manual. Adequate target cells can be prepared without use of Lympho-Kwik™ by using combinations of these preparative procedures. Because of its simplicity and consistently good results, however, use of this reagent is recommended.

Similarly, there are variations in making single cell suspensions from node and spleen tissue. These include cutting the tissue into small pieces and teasing or scraping cells apart with a needle or scalpel tip; applying pressure to the tissue with the flat edge of a scissors or scalpel; pressing the tissue through metal screening (such as a tea strainer); or vigorous shaking or stirring of spleen fragments suspended in medium. Larger pieces of tissue are removed by allowing them to settle out of suspension, or sieving through gauze pads. We have used some of these techniques but are much more satisfied with the resultant cell preparation when milder conditions, as described above, are employed.

Limitations of Procedures

1. Viability is always a problem.
2. Very, very rarely, no cells are obtained from the lymph nodes.

In either case, more materials may be requested or one may revert to peripheral blood as the lymphocyte cell source if such has been provided.

References

1. Biegel AA, Heise ER, MacQueen JM, Schacter B and Ward FE, Cell preparation and preservation for cytotoxicity testing. In: *SEOPF Tissue Typing Reference Manual*, JM MacQueen, ed.; South-Eastern Organ Procurement Foundation, Richmond; p I-1, 1976.
2. Garcia ZC and Gal K, Cell preparation. In: *Tissue Typing Reference Manual 1987*, JM MacQueen and G Tardif, eds.; South-Eastern Organ Procurement Foundation, Richmond; p C11-1, 1987.
3. Weaver P and Cross D, Isolation of lymphocytes from lymph nodes. In: *AACHT Laboratory Manual*, AA Zachary and WE Braun, eds.; American Association for Clinical Histocompatibility Testing, New York; p I-4-1, 1981.
4. Weaver P and Cross D, Isolation of lymphocytes from spleen. In: *AACHT Laboratory Manual*; AA Zachary and WE Braun, eds.; American Association for Clinical Histocompatibility Testing, New York; p I-5-1, 1981.

Immunomagnetic Isolation of Lymphocyte Subsets Using Monoclonal Antibody-Coated Beads

Julia A. Hackett and Nancy F. Hensel*

Purpose

Immunomagnetic beads coated with a specific monoclonal antibody (e.g., anti-CD2, anti-CD8, anti-CD19) are added to a cell suspension containing the target cells (e.g., CD2+, CD8+, CD19+). During a short incubation period, the beads bind to the target cells (positive selection), and the rosetted cells can be isolated by the use of a magnetic device. The isolated lymphocyte subset (purity and viability >95%) may be washed and used for HLA typing procedures as well as in crossmatching.

Specimen

A suspension of unseparated mononuclear cells, either fresh or frozen (see Procedure Note #8). Blood collected in sodium heparin is not recommended unless the isolation from whole blood is performed within twelve hours after collection. If acid citrate dextrose (ACD) or citrate phosphate dextrose adenine (CPDA) is used, isolation should be performed within three days.

CAUTION: The cell suspension must contain cells that express the target surface antigen (e.g., CD2, CD8, CD19). Cell samples with poor viability (<80%) should be treated with DNAse (or other preparation such as Lympho-Kwik™ [One Lambda], Revive-a-Cell [Gen Trak]) prior to performing the separation.

Reagents and Supplies

1. Phosphate Buffered Saline (PBS), 1X, pH 7.2, no Ca^{++} or Mg^{++}.
   a. Cat. No. P310-500, Biofluids, Inc., Rockville, MD.
   b. Store at room temperature.
2. Monoclonal Antibody Coated Magnetic Beads. For example, Dynal Dynabeads®, One Lambda Fluorobeads® B, Biotest Lymphobeads.
   a. CD2 (Pan-T) Dynabeads® M-450.
      1) Cat. No. 111.01, Dynal A.S., Oslo, Norway.
      2) Store at 2° C-8° C.
   b. FluoroBeads-B.
      1) Cat. No. FB2-25, One Lambda, Inc., Canoga Park, CA.
      2) Store at 2° C-8° C.
   c. Lymphobeads HLA Class I.
      1) Cat. No. 824120, Biotest Diagnostics Corp., Denville, NJ.
      2) Store at 2° C-8° C.
3. RPMI 1640 or other culture media with 2% heat-inactivated fetal bovine serum.
   a. RPMI 1640 with glutamine, pH 7.1-7.2.
      1) Cat. No. 102G-500, Biofluids, Inc., Rockville, MD.
      2) Store at 4° C.
   b. Fetal bovine serum, heat-inactivated (FBS).
      1) Cat. No. 200C-100, Biofluids, Inc., Rockville, MD.
      2) Store at –20° C.
4. Stock Solution of acridine orange/ethidium bromide dye (AO/EB):
   a. Acridine Orange.
      1) Cat. No. 15,855-0, Sigma-Aldrich, St. Louis, MO.
      2) Health hazard: carcinogen.

* Supported by the National Institutes of Health (NIH). Views presented in this paper are those of the authors; no endorsement by the NIH has been given or should be inferred.

b. Ethidium Bromide powder or Ethidium Bromide tablets (10 x 11 mg/vial) .
   1) Cat. No. E8751 Sigma-Aldrich, St. Louis, MO (powder).
   2) Cat. No. 161-0430, BioRad, Hercules, CA (tablets).
   3) Store at room temperature in the dark
   4) Health hazard: carcinogen
c. Dissolve 15 mg AO and 50 mg EB in 1 ml ethanol. Add 49 ml PBS. Store at 4° C (up to one week) or aliquot and freeze at –20° C. Stable when frozen for at least six months.

5. Hemoglobin Quench:
   a. Lyophilized bovine hemoglobin.
      1) Cat. No. H2625, Sigma-Aldrich, St. Louis, MO.
   b. 2% EDTA-PBS.
      1) EDTA, Cat. No. E-5016, Sigma-Aldrich, St. Louis, MO.
   c. 1% Sodium Azide.
      1) Cat. No. S-227, Fisher Scientific, Fair Lawn, NJ.
   d. Dissolve 10 g lyophilized bovine hemoglobin in 100 ml 2% EDTA-PBS. Add 1 ml 1% sodium azide. Centrifuge at 1000 x g for 30 min. Decant supernatant and freeze at –20° C. Can be stored at 4° C for one week.
6. AO/EB-Hemoglobin Reagent:
   a. Mix 6.5 ml Hemoglobin Quench, 3.5 ml deionized water and 200 µl stock AO/EB. Store at 4° C for one week.

Instrumentation/Special Equipment

1. Magnetic Separator, Rare Earth.
   a. Dynal MPC-6, Cat. No. 12002, Dynal A.S., Oslo, Norway.
   b. FluoroBeads Magnet, Cat. No. FBA-MAG, One Lambda, Inc., Canoga Park, CA.
   c. Biotest Magnet MSD, Cat. No. 824130, Biotest Diagnostics Corp., Denville, NJ.
2. Apparatus for rotation of test tubes.
   a. Dynal® Sample Mixer, Cat. No. 947.01, Dynal A.S., Oslo, Norway.
3. Refrigerator or cold room.

Calibration

Not applicable.

Quality Control

1. Each new container of beads should be washed according to manufacturer's instructions to remove free antibody that could reduce cell yield.
2. Prior to use in testing, every new lot of antibody coated beads should be tested in parallel with a previous or acceptable lot of beads to determine that quality and quantity of isolated cells is adequate. This parallel testing must be documented.
3. Standard reagents and equipment QC procedures should be performed and must be documented. In particular, refrigerator temperatures should be verified.

Procedure

1. If frozen cells are used, thaw according to standard operating procedure. If fresh cells are used, isolate mononuclear cells from whole blood according to standard operating procedure.
2. Resuspend the cell pellet in 1.0 ml chilled PBS and place in wet ice.
3. Perform a cell count and viability. If the viability is less than 80%, the cell suspension should be treated with DNAse[6] or Lympho-Kwik™ before performing the separation.
4. The volume of magnetic beads to be used is determined by the cell count according to Table 1.
5. To a labelled 1.5 ml snap cap microcentrifuge tube add 1 ml chilled PBS and the appropriate volume of well mixed magnetic beads.
6. Mix well and place uncapped on the magnetic separator for 1 min. Discard supernatant.
7. Remove the tube from the magnet, add the cell suspension, and cap tightly.
8. Immediately place on rotator in the refrigerator or the cold room. **The temperature should be less than 8º C.** Rotate for exactly 5 min at slow to medium speed.
9. Remove the tube from the rotator, uncap and place on the magnetic separator. If possible, this step should be done in the cold. Leave tubes undisturbed for 3 min.
10. Transfer the target cell-depleted supernatant into another labeled tube or discard if not needed.
11. Remove the tube from the magnet. Add 1 ml chilled PBS, cap and gently invert to resuspend the rosetted beads.
12. Uncap and place on the magnetic separator for 1 min. Discard supernatant.

13. Repeat steps 11 and 12.
14. Remove tube from the magnet and resuspend the rosetted beads in media according to Table 2.
15. Evaluate the cell count and viability using acridine orange/ethidium bromide (AO/EB); alternatively the 1-2-3 drop technique can be used to evaluate cell count and viability (see Procedure Note #1).

Table 1

Cell Count (x 10^6)	Volume of Beads (µl)
<10	20
10-20	25
21-30	30
31-40	35

Table 2

Initial Count (x 10^6)	Volume of Media (µl)	
	B Cells	T Cells
<10	150	300
10-20	300	400
21-30	400	500
31-40	500	700

Calculations

Not Applicable.

Results

Expect to obtain a subset of lymphocytes diluted to the desired concentration with a viability and purity of greater than 80%.

Procedure Notes

1. 1-2-3 Drop Technique[4]

a. To a clean Terasaki tray add:
   1) 1 µl of the cell suspension to the 1st well
   2) 2 µl of the cell suspension to the 2nd well
   3) 3 µl of the cell suspension to the 3rd well

b. Add 5 µl of the AO/EB hemoglobin dye to each well.

c. Observe under the fluorescence scope and choose the well with the best cell concentration.
   1) 1st well: Cell concentration is correct; proceed with plating.
   2) 2nd well: Reduce the volume of the cell suspension by 1/2.
   3) 3rd well: Reduce the volume of the cell suspension by 2/3.

2. ABC and DR Typing

The standard NIH method can be used with standard incubation times. Instead of using eosin or trypan blue, fluorescent dyes are used. The fluorescent dye is added to the complement (50µl AO/EB per ml complement). Usually the dyes used are acridine orange/ethidium bromide or carboxylfluorescein diacetate (CFDA)/propidium iodide (PI). With either combination of dyes, live cells will appear green and dead cells appear orange-red. In order to easily visualize the cells, a hemoglobin-EDTA quench is added at the end of the complement incubation. The EDTA chelates the free calcium ions so that complement activity ends. The hemoglobin provides a dark background so that the cells are easily seen. India ink can also be used as a quenching reagent.

3. Fluorescent Dyes

a. CFDA and acridine orange (AO) are used to indicate live cells. The advantage of CFDA is that the cells can be stained prior to being used in the cytotoxicity assay. Once the dye enters the cell, an ester group is cleaved which prevents the dye from diffusing out the cell membrane. A quenching agent is not needed to visualize the cells since the dye is contained within the cell membrane. The fluorescent emission of CFDA is much stronger than AO therefore the cells appear brighter when stained with CFDA than with AO.

b. AO freely crosses cell membranes and is not retained by the cell once inside the membrane. A quenching agent like hemoglobin or India ink is needed to distinguish the stained cells. AO does not require a separate staining step since it can be added with the ethidium bromide to the complement or to the quench-EDTA reagent.

c. Both propidium iodide and ethidium bromide are vital dyes meaning they do not cross intact membranes. The two dyes are very similar in fluorescent intensity and emission wavelength.
d. Acridine orange, ethidium bromide and propidium iodide are considered carcinogens and therefore must be handled with extreme care.

4. Quenching Reagents

Hemoglobin and India ink are commonly used as quench reagents.

a. Hemoglobin (bovine or human can be used) gives uniform background and provides protein to extend cell viability so that trays can be read up to three days after preparation. Hemoglobin preparation is more time-consuming than India ink.
b. India ink tends to settle in the wells, and physically blocks light coming through the well making it difficult to visualize the cells. Trays using ink as a quench cannot be stored before reading without significant loss in viability of the cells.

5. Inverted Fluorescence Microscope

A 100 watt high pressure mercury lamp is recommended. Xenon can also be used. The combination of excitation, emission filters and dichroic mirror is important. The Nikon B-2A filter block works very well; i.e., Excitation 450-490 nm, dichroic mirror 510 nm, emission 520 nm (long pass filter).

6. DETACHaBEAD

In certain cases, if a highly pure bead-free cell population is required, the product DETACHaBEAD (Dynal Inc.) can be used to free the cells from the beads. This product is an ammonium sulfate precipitated Fab IgG antibody made by immunizing sheep or goats with Fab fragments of papain digest of mouse immunoglobulin. It reacts with the mouse IgG antibody on the bead, freeing the cell from the bead. The cells are not activated and have all surface antigens intact and fully functional. Used according to insert instructions, from 1 to 10 x 10^6 cells can be successfully harvested from Dynabeads if the bead-cell ratio is from 3-10. Detachment of cells from other monoclonal coated beads may be successful but the efficiency of detachment is not guaranteed by the manufacturer.

7. Troubleshooting

a. Staining problems with certain cells:
AO binds to RNA as well as DNA. When AO binds to RNA, it fluoresces at a wavelength similar to ethidium bromide and propidium iodide. In normal lymphocytes, there is usually no significant binding to RNA in the cytoplasm but problems can occur in stimulated lymphocytes which are manufacturing protein; instead of either red or green cells, there will be cells stained red and green. An alternative stain such as CFDA will eliminate this problem. Also, a one-color technique can be used with ethidium bromide or propidium iodide in which case only the dead cells will be visible.
b. Fluorescent intensity too low:
   1) If green is too pale, increase the concentration of CFDA or AO.
   2) If red is too pale, increase the concentration of EB or PI.
   3) Decrease the concentration of ink.
   4) Increase fluorescence light source from 50 watts to 100 watts.
c. Fluorescent intensity too high:
   1) If the background fluorescence is too intense, increase the concentration of the quench reagent.
   2) If the green is too intense, decrease the concentration of CFDA or AO.
   3) If the red is too intense, decrease the concentration of EB or PI.
   4) Reagents may be contaminated with bacteria or mycoplasma; make new reagents.
d. Low viability of cells on typing trays:
   1) If cell viability is determined by trypan blue, the viability will appear to be decreased on the trays. Ethidium bromide and propidium iodide molecules are much smaller than trypan blue or eosin molecules. Thus, complement lysis will seem enhanced since a smaller degree of injury to the cell will result in the EB or PI crossing the cell membrane.
   2) Monocyte or granulocyte contamination. Use Lympho-Kwik™, carbonyl iron or thrombin before performing the bead separation.
   3) Isolation not performed within the time specified for the anticoagulant. Treat the cell prep with DNAse or Lympho-Kwik™ before performing the bead separation. Alternatively, the bead-cell rosettes can be treated with DNAse to improve the viability before plating; however, the bead:cell ratio will be markedly increased making tray reading difficult.
e. Weak reactivity:
   1) Ensure adequate mixing of cells with sera.
   2) Try an increase in the incubation time.
   3) Make sure the cell concentration is not too heavy. 1.0-1.5 x 10^6 cells/ml is adequate.

8. Whole Blood Samples

Protocols for the use of whole blood samples are available from several commercial sources (Dynal, Inc., Biotest, Inc., One Lambda,Inc.).

Limitations of Procedure

Optimum yields are dependent on proper expression of the target antigen on the surface of the cell. Certain disease states (e.g., leukemia) as well as the effect of immunosuppressive drug therapy may result in the down-regulation of certain cell surface antigens.

References

1. Biotest Product Insert for Lymphobeads HLA Class I and II.
2. DYNAL™ Product Insert for DETACHaBEAD.
3. DYNAL™ Product Insert for DYNABEADS™ HLA Cell Prep I and II.
4. One Lambda, Inc. Product Insert for FluoroBeads®B, Rev.11/24/92.
5. One Lambda, Inc. Product Insert for Lympho-Kwik®, Rev. 3/90
6. Strong DM. Cryopreservation of Lymphocytes in Bulk. In: *ASHI Laboratory Manual*, 2nd Edition, A. Zachary and G. Teresi, eds., American Society for Histocompatibility and Immunogenetics, Lenexa, pp. 158-163, 1990.

Nylon Wool Separation of T and B Lymphocytes

Marilena Fotino and Arvind K. Menon

Purpose

To separate T and B lymphocytes for HLA typing, antibody screening and crossmatching. The nylon wool separation of B lymphocytes is based on the empirical observation that B lymphocytes adhere preferentially to nylon wool from which they can be eluted, whereas T lymphocytes do not adhere. B lymphocyte adherence to nylon is an active process and is reduced at 20° C or 4° C and by the presence of sodium azide or EDTA.[3, 6] The main advantages of the nylon wool column separation of B and T lymphocytes lie in the simplicity of the technique and the short time necessary to obtain the two cell populations. B lymphocytes eluted from nylon wool columns have excellent viability (95%) and are virtually free of monocytes. At the same time, since the technique does not require any agent which would interfere with the cell surface, the lymphocytes are not exposed to any antigens, enzymes or antisera.

Specimen

Acceptable

Separated lymphocytes with >80% viability.

Unacceptable

Lymphocytes with <80% viability

Reagents and Supplies

1. Phosphate buffered saline (PBS)
2. Hank's balanced salt solution (HBSS)
3. McCoy's
4. RPMI 1640
5. Heat-inactivated fetal calf serum (HIFCS)
6. Deoxyribonuclease (DNAse): 2.75 mg/ml; 100 ml aliquots in Beckman tubes stored at -80° C.
7. Bovine serum albumin (BSA): 100 NIH units/ml saline solution. For sterile preparation, filter through 0.45 m or 0.2 m filter. Store below 0° C.
8. Trypan blue solution: 10 ml trypan blue, 2% in H_2O + 85 ml PBS + 5 ml 5% Na EDTA in PBS.

Instrumentation/Special Equipment

1. 37° C incubator
2. 37° C water bath
3. Centrifuge
4. Upright microscope
5. Refrigerator
6. Coulter counter or hemocytometer
7. Analytical balance

Calibration

Standard calibrations for centrifuge rotor speed, all thermometers and temperature regulated equipment, incubator percent CO_2, and microscopes should be performed and must be documented. Centrifuge and rotor should be capable of reaching appropriate speeds, generating appropriate g forces, and containing appropriate sized tubes.

Quality Control

Viability Control

Viability of the separated lymphocytes is checked by trypan blue (see Assessment of Cell Preparations I.A.12).

Purity Control

Purity of the B and T lymphocytes is assessed by using complement dependent lymphocytotoxic sera reacting with T cells, B cells, monocytes, etc. (see Assessment of Cell Preparations I.A.12).

Procedures

Nylon Wood Straw Microtechnique

The proportions given below are for a column[1] able to handle up to 10 x 10^6 cells.

1. Heat seal one end of a flexible, transparent drinking straw (0.6 x 12-14 cm) at a 45° angle.
2. Thoroughly tease 0.1 g of scrubbed nylon wool while soaking in HBSS or PBS in a Petri dish.
3. Fill ¾ of the straw with HBSS or PBS, then, using the tip of a pipette, gradually and evenly pack the nylon wool into the straw to a height of approximately 6 cm. At this stage the column can be stored at 4° C for up to 2 weeks.
4. Cut or puncture the sealed end of the straw to make an opening of approximately 2 mm.
5. Flush the nylon wool with 5 ml HBSS or PBS and then with 5 ml medium containing 5% HIFCS.
6. When the medium just covers the nylon wool, turn the straw to a horizontal position and incubate 30 min at 37° C. Alternatively, use prewarmed medium.
7. Add 0.5 ml of purified lymphocyte suspension (5-20 x 10^6 cells/ml) in 5% HIFCS to the top of the column and allow the cells to move all the way into the wool. A good T and B cell separation depends on the purity of the initial lymphocyte preparation. Therefore, the suspension should be devoid of granulocytes and platelets.
8. Add approximately 0.2 ml 5% HIFCS to the top of the column to prevent drying. Lay the column flat and incubate 30 min at 37° C.
9. To recover T lymphocytes, allow 2 washes (8 ml each) of warm (37° C) 5% HIFCS to drip through the column held vertically.[5] The effluent contains non-adherent T cells.
10. To recover the adherent B cells, add 1.5 ml 5% HIFCS to the column and repeatedly squeeze the straw. Continue this step until 8 ml of medium have been used.
11. Centrifuge both T and B cell suspensions 5 min at 1000 x g and wash once with 1 ml 5% HIFCS.
12. Resuspend cells in a minimum amount of medium (e.g. 0.5 ml), check viability, count the cells and adjust the concentration to 2 x 10^6 cells/ml.

On the average, this procedure should provide recovery of 80-90% of the cells.

Nylon-on-a-Stick Method

1. Suspend the lymphocytes used for separation in 0.5 medium/5% HIFCS.[8, 9]
2. Put 0.1 g of brushed nylon wool into a 17 x 100 mm plastic tube.
3. Fill the tube with 5% HIFCS and centrifuge at 320 x g for 3 min to remove air bubbles and thoroughly wet the nylon wool (the tubes can be frozen at this stage and stored).
4. Incubate the nylon wool at 37° C for 30 min.
5. Insert an applicator stick down the side of the tube with nylon wool and twirl the nylon wool onto the stick about ½ inch above the end of the stick. Remove the nylon wool-covered stick slowly, expressing most of the medium on the side of the tube.
6. Slowly drip 0.5 ml of the lymphocyte suspension into the nylon wool stick held above a 50 ml conical plastic tube. Use parafilm to seal and hold the stick upright.
7. Incubate the tube vertically at 37° C for 30 min.
8. Elute the non-adherent T cells by washing the nylon wool stick with 10 ml warm (37° C) 5% HIFCS.
9. Repeat the process into a second tube which will contain a mixture of T and B cells.
10. Place the nylon wool stick in a 17 x 100 mm plastic tube containing 10 ml 5% HIFCS.
11. Dislodge the adherent B cells by vigorous twirling of the nylon wool and compressing against the wall of the tube.
12. Centrifuge the non-adherent and adherent cells, wash and adjust the cells with medium to the desired concentration.

Nylon-Column Syringe Method

Recommended for separating larger amounts of lymphocytes (>20 x 10^6/ml).[7, 9]

1. Tease apart 0.3-0.4 g of nylon wool and loosely pack it into a 5 ml plastic syringe.
2. Affix a 3-way plastic stopcock with 18 gauge needle to control flow through the syringe.
3. Pour 5% HIFCS into column until the nylon wool is wet. With stopcock in the off position, fill syringe with 5% HIFCS.
4. Cover top of syringe with parafilm and place in a 37° C incubator for 30 min. Separately warm approximately 30 ml of 5% HIFCS.
5. Take the warmed column from the incubator and allow the top portion of the 5% HIFCS to run off into the nylon wool, using the stopcock for control.
6. Add 1 ml of 5% HIFCS containing 20-60 x 10^6 cells to the column. Using the stopcock, allow the cell suspension to enter the nylon wool fibers, stopping just before the fluid escapes. Top off the remaining space in the syringe with warmed 5% HIFCS, seal with parafilm and incubate at 37° C for 30 min.[9]

7. Mark 17 x 100 mm plastic tubes "T Cells" and "B Cells".
8. To recover the T lymphocytes allow 10-20 ml of warm 5% HIFCS to drip through the syringe held vertically into the tube. The effluent contains the T cells (non-adherent to the column). Be careful not to jar the column to avoid detaching the B cells from the nylon wool.
9. To recover the adherent B lymphocytes, add to the syringe 10 ml of cool (10° C) medium without HIFCS (which enhances the adherence of the B cells to the nylon wool). While medium is added, detach the B cells by poking the nylon wool with a pipette or applicator stick and collect the effluent. Squeezing the nylon wool with the syringe plunger is not recommended, as it may release monocytes which would increase the background of dead cells in the test.
10. Centrifuge the T and B cell enriched suspensions of lymphocytes (320 x g for 10 min).
11. Discard the supernatants and adjust the cells with medium to the desired concentration.

Calculations

Not applicable

Results

Reference Ranges

The isolation techniques given use lymphocyte preparations obtained from peripheral blood, spleen or lymph nodes, the latter two giving a higher yield of B cells. On the average, the nylon wool procedures provide a recovery of 80-90% of the cells[1]. From 20 ml of normal blood the B cell yield ranges from 1-1.5 x 10^6 cells.[5]

Procedure Notes

Troubleshooting

The most common problems encountered are contamination of the B lymphocyte suspension with polynuclear cells, poor lymphocyte separation and poor viability. The elimination of polynuclear cells from the B cell suspension can be achieved by various methods: differential centrifugation, carbonyl iron and thrombin (see the corresponding chapters).

Thrombin Removal of Polynuclear Cells[10]

1. To the platelet and/or granulocyte contaminated lymphocyte suspension, add 1 drop of autologous plasma and 1 drop of thrombin (100 units/ml of saline) and mix.
2. Rotate the tube (8 revolutions/min) for 2-5 min, or until small clumps are formed.
3. After a 3 second (on/off) centrifugation at 1000 x g, collect the supernatant containing purified lymphocytes.
4. Centrifuge at 1000 x g for 1 min and wash the pellet with PBS.

Poor Viability

The removal of dead lymphocytes can be obtained by serum albumin flotation and/or DNAse treatment.

DNAse Treatment

1. Add 0.4 ml DNAse stock solution to 1 ml lymphocyte suspension (5-10 x 10^6 cells) and incubate 5 min in a 37° C water bath. Mix occasionally by pipetting.
2. Wash the cells twice with HBSS containing 5% HIFCS.
3. Centrifuge for 3 seconds at 1000 x g in a Fisher Model 59 centrifuge to eliminate debris. Recheck cell suspension for viability and concentration.

Serum Albumin Flotation

This technique is based on the lower density of any dead cells present.

1. Layer the cell suspension over 0.4 ml BSA medium (6 parts 30% BSA + 5 parts HBSS) and centrifuge for 1 min at 1000 x g. Dead cells remain at the interface while live cells settle to the bottom of the tube.
2. Discard the supernatant and resuspend the pellet of live cells in HBSS.
3. Wash the cells twice. Recheck the cell suspension for viability and concentration.

Inadequate Separation

The contamination of B cells with T cells which usually occurs when the nylon wool column is not sufficiently washed prior to extracting the B lymphocytes, can be avoided by giving an extra wash with 5-8 ml of medium.

The T cell contamination with B cells is encountered when the nylon wool is not properly teased to offer a large surface for the attachment of B cells. Thorough teasing of the nylon wool while soaking in PBS prior to packing it into the column results in a better separation.

Monocyte Contamination

Monocyte contamination can be avoided by treating the lymphocytes with carbonyl iron prior to adding them to the nylon wool column. In stubborn cases repeating the iron treatment of the separated B cells can help (see Cell Separation chapter).

Limitation of Procedure

Not applicable

References

1. Danilovs JA, Ayoub G, andTerasaki P, B lymphocyte isolation by thrombin-nylon wool. In: *Histocompatibility Testing*, PI Terasaki, ed, UCLA Tissue Typing Laboratory, Los Angeles, p 287, 1980.
2. Dupont B, Jersild C and Jakobson B, Elimination of non-viable cells by DNAse treatment prior to lymphocytotoxicity tests. Tissue Antigens 2:141, 1972.
3. Eisen SA, Wedner HJ, Parker CW, Isolation of pure human peripheral blood T lymphocytes using nylon wool columns. Immunol Commun 1:571, 1972.
4. Fotino M, Nylon wool separation of T and B lymphocytes. In: *American Society for Histocompatibility and Immunogenetics Laboratory Manual* 2nd edition: AA Zachary and G Teresi, eds., American Society for Histocompatibility and Immunogenetics, Lenexa p 65, 1990.
5. Fotino M and Menon AK, Nylon wool separation of T and B lymphocytes. In: *AACHT Laboratory Manual*; AA Zachary and WE Braun eds., AACHT, NY, p I-6-1, 1981
6. Greaves MF, and Brown G, Purification of human T and B lymphocytes. J Immunol 122:420, 1974.
7. Lowry R, Goguen J, Carpenter CB, Strom TB, and Garovoy MR, Improved B cell typing for HLA-DR using nylon wool enriched B lymphocyte preparations. Tissue Antigens 14:325, 1979.
8. Severson C and Thompson J, Nylon B-Cell isolation from EDTA blood. Workshop Newsletter No. 6, Eighth International Histocompatibility Workshop, Los Angeles, 1980.
9. Tardif GN, B-cell isolation with nylon In: *Tissue Typing Reference Manual*; JM MacQueen, GN Tardif, eds: SEOPF, Richmond, p C18:1, 1987.
10. Terasaki PI, Park MS, Loon J, and Bernoco D, *UCLA Tissue Typing Manual*, 1987.

Isolation of T Lymphocytes: A Quick Mini Method for Small Sample Sizes

Afzal Nikaein

Purpose

To obtain a viable highly purified T lymphocyte subset suspension for use in HLA typing and crossmatching procedures.

Specimen

Acceptable Specimen

Whole blood or a suspension of unseparated mononuclear cells, either fresh or frozen. Acid citrate dextrose (ACD) and citrate phosphate dextrose adenine (CPDA) are the preferred anticoagulants and may be used for blood up to 5 days old for Class I HLA typings only.

Unacceptable Specimen

Cells not expressing the target surface antigen (for example: CD2, CD8, CD19). Cell samples with poor viability (<80%) should be treated with DNAse (or other equivalent preparation such as Lympho-Kwik™ [One Lambda], Revive-a-Cell [Gen Trak]) prior to performing the separation.

Reagents and Supplies

1. Phosphate Buffered Saline (PBS), no Ca^{++} or Mg^{++}
2. Monoclonal Antibody Coated Magnetic Beads (Dynal Dynabeads, Biotest Lymphobeads, One Lambda Fluorobeads® T)
3. Developer (if Fluorobeads® T are used).
4. 5 ml glass tubes

Instrumentation/Special Equipment

1. Magnetic Separator, preferably a Rare Earth magnet
2. Apparatus for rotation of test tubes
3. Refrigerator or cold room
4. Calibrated micropipettes or Hamilton syringes

Calibration

Not Applicable

Quality Control

No specialized control procedures other than the standard reagent and equipment control procedures. These must be followed and documented.

Procedure

Table 1. Estimation of Required Test Volume of Beads Based on Initial Cell Count

Cell Count (x 10^6)	Volume of Beads (µl)
<10	20
10-20	25
21-30	30
31-40	35

1. If frozen cells are used, thaw according to standard operating procedure.
2. Resuspend the cell pellet in 1.0 ml RT PBS.
3. Perform a cell count and viability. If the viability is less than 80%, the cell prep should be treated with DNAse or Lympho-Kwik™ before performing the separation.
4. For whole blood, add 1.0 ml of blood to an empty glass tube. Label the tubes in advance.
5. Add 50 to 100 µl of magnetic beads. The volume depends on the number of cells to be obtained & number of HLA typing trays to be used. See Table 1 above.
6. Immediately place on rotator in the refrigerator or the cold room(for Dynabead or Biotest Lymphobeads) and at RT (for Fluorobeads® T). Rotate for exactly 5 min at slow to medium speed.
7. After 5 min, remove the tubes from the rotator, uncap and place on the magnetic separator. For Fluorobeads® T, add 0.5 ml Developer, gently shake the tubes & place them on the magnets. This step should be done at RT. Leave tubes undisturbed for 5 min.
8. After 5 min, aspirate the target cell depleted supernatant or discard by holding on to the tubes & pouring the supernatant out.
9. Remove the magnet from the holder. Add 1 ml RT PBS.
10. Insert the magnet all the way into the magnet holder, leave it at RT for 1 min.
11. Follow step 8 above.
12. Repeat steps 9-11.
13. Remove tube from the magnet and resuspend the beads in 3-5 drops of RT PBS. With our experiences PBS results in better viability if the isolated cells are used on the same day.
14. Evaluate the cell count and viability using acridine orange/ethidium bromide (AO/EB); alternatively the 1-2-3 drop technique for cell counting can be used. See instructions below.

****

1-2-3 Cell Counting Procedure

1) To a clean Terasaki tray add:
   1 µl of the cell suspension to the 1st well
   2 µl of the cell suspension to the 2nd well
   3 µl of the cell suspension to the 3rd well
2) Add 5 µl of the AO/EB Quench dye to each well
3) Observe under the fluorescence scope and choose the well with the best cell concentration.
   1st well: if cell concentration is correct; proceed with plating.
   2nd well: reduce the volume of the cell suspension by 1/2.
   3rd well: reduce the volume of the cell suspension by 2/3.

****

Results

Expect to obtain enough cells for 1 to 2 Class I typing trays.

Procedure Notes

1. **ABC Typing.** The standard NIH method can be used with standard incubation times. Instead of using eosin or trypan blue, fluorescence dyes are used. Live cells will appear green and dead cells will appear orange-red.
2. **Fluorescence Dyes.** Any fluorescent dye used in your laboratory may be adapted to this technique. CFDA and acridine orange (AO) are used to indicate live cells. The advantage of CFDA is that the cells can be stained prior to being used in the cytotoxicity assay (once the dye enters the cell, an ester group is cleaved which prevents the dye from diffusing out the cell membrane.). A quenching agent is not needed to visualize the cells since the dye is contained within the cell membrane. The fluorescence emission of CFDA is much stronger than AO therefore the cells appear brighter when stained with CFDA than with AO.
3. AO freely crosses cell membranes and is not retained by the cell once inside the membrane. A quenching agent like hemoglobin or India ink is needed to distinguish the stained cells. AO does not require a separate staining step since it can be added with the ethidium bromide to the complement or to the quench-EDTA reagent.
4. Both propidium iodide and ethidium bromide are vital dyes meaning they do not cross intact membranes. The two dyes are very similar in fluorescence intensity and emission wavelength.
5. Acridine orange, ethidium bromide and propidium iodide are considered carcinogens and therefore must be handled with extreme care.
6. **Quenching Reagents.** Hemoglobin and India ink are commonly used as quench reagents. Hemoglobin (bovine or human can be used) gives uniform background and provides protein to extend cell viability so that trays can be read up to three days after preparation. Hemoglobin preparation is more time-consuming than India ink.
   India ink tends to settle in the wells, and physically blocks light coming through the well making it difficult to visualize the cells. Trays using ink as a quench cannot be stored before reading without significant loss in viability of the cells.

7. **DETACHaBEAD.** In certain cases, if a highly pure bead-free cell population is required, the product DETACHaBEAD (Dynal Inc.) can be used to free the cells from the beads. This product is an ammonium sulfate precipitated Fab IgG antibody made by immunizing sheep or goats with Fab fragments of papain digest of mouse immunoglobulin. It reacts with the mouse IgG antibody on the bead, freeing the cell from the bead. The cells are not activated and have all surface antigens intact and fully functional. Used according to insert instructions, from 1 to 10 x 10^6 cells can be successfully harvested from Dynabeads if the bead-cell ratio is from 3-10. Detachment of cells from other monoclonal coated beads may be successful but the efficiency of detachment is not guaranteed by the manufacturer.

Troubleshooting

1. Low viability of cells on typing trays:
   If cell viability is determined by trypan blue, the viability will appear to be decreased on the trays. Ethidium bromide and propidium iodide molecules are much smaller than trypan blue or eosin molecules. Thus, complement lysis will seem enhanced since a smaller degree of injury to the cell will result in the EB or PI crossing the cell membrane.
2. Monocyte or granulocyte contamination. Use Lympho-Kwik™, carbonyl iron or thrombin before performing the bead separation.
3. Isolation not performed within the time specified for the anticoagulant. Treat the cell prep with DNAse or Lympho-Kwik™ before performing the bead separation. Alternatively, the bead-cell rosettes can be treated with DNAse to improve the viability before plating; however, the bead:cell ratio will be markedly increased making tray reading difficult.
   If weak reactivity occurs, first ensure adequate mixing of cells with sera.
   Try an increase in the incubation time to strengthen the reaction.
4. Make sure the cell concentration is not too heavy. 1.0-1.5 x 10^6 cells/ml is adequate.

Calculations

Normal cell counting procedures are required for this test.

Limitations of Procedure

1. If the lymphocyte cell count is low, try starting with 2 ml of whole blood.
2. This microtest can be used only for T cells. For B lymphocytes, the whole buffy coat is required.

References

1. One Lambda, Inc. Product Insert for FluoroBeads®B, Rev.11/24/92.
2. Biotest Product Insert for Lymphobeads HLA Class I and II.
3. DYNAL™ Product Insert for DYNABEADS™ HLA Cell Prep I and II.

Rosetting as a Method for Separating Human B Cells and T Cells

Dod Stewart and Sue Herbert

Purpose

Separation of human B cells and T cells by rosetting is commonly performed using sheep red blood cells (SRBC). However several modifications of this method are also in use and are presented as detailed procedure variations following the standard procedure. These include: Neuraminidase-treated SRBC, AET-treated SRBC, rosetting in the presence of Dextran, anti-human-FAB-coated SRBC and rosetting with Ox RBC and monoclonal antibody.

Rosetting with Sheep Red Blood Cells

Purpose

Human peripheral blood T Cells can be identified by the formation of E-rosettes with sheep erythrocytes. This phenomenon is mediated by receptors on the T cells specific for the sheep red blood cells (SRBC's). It is now recognized that the CD2 marker on T cells is the SRBC receptor.[10, 12] This property, under normal conditions, is not shared by monocytes, granulocytes or B lymphocytes, hence, the method described herein can serve as a basis for separating T cells from other peripheral blood mononuclear cells.[8, 11, 28]

The relative simplicity of this rosetting technique resulted in its wide practical application. However, other simpler methods have limited the use of the technique for T cell isolation. Because of the difference in density, rosetted and non-rosetted cells can be separated by density gradient centrifugation. Since this procedure allows for the recovery of both the E-rosetted T cells and the non-E-rosetted population, this method can be utilized for obtaining purified B cells, provided that the monocyte cellular component of the peripheral blood mononuclear cell (PBMC) fraction obtained following Ficoll-Hypaque (FH) density gradient centrifugation is eliminated or reduced prior to the E-rosetting procedure.[3] Monocyte depletion is an important step in the isolation of purified B cells by E-rosetting since monocytes constitute about 20-30% of the PBMC obtained by density gradient centrifugation.

Modifications of the original rosetting method have arisen to minimize variability or to reduce the amount of time necessary to complete the separation. A description of some of the most common variations are included. The E-rosetting procedures of Stux, et al.,[22] follow.

Specimen

Caution: Because human blood or tissue is used in this procedure, appropriate laboratory technique must be followed. Handle all samples as if capable of transmitting disease.

Acceptable

Any whole lymphocyte population isolated by methods described in the lymphocyte isolation section of this manual.

Unacceptable

Whole lymphocyte populations that are less than 80% viable.

Reagents/Supplies

1. Labels
   All reagents must be properly labeled to indicate:
   a. Identity
   b. Titer, strength, or concentration, when significant
   c. Preparation and/or expiration date
   Storage requirements, or other pertinent information: Reagents must be stored according to manufacturers' instructions, at temperatures appropriate to maintaining reactivity and specificity. Reagent performance must be checked before placing the reagent in service.

2. Sheep Erythrocytes
   a. Maintain commercially obtained SRBC in Alsever Solution at 4ºC. These cells can be used up to 3-4 weeks following their arrival.

   Prepare sheep erythrocytes fresh, either daily, or every other day.
   b. Wash 2 ml of SRBC four times in 10-20 ml isotonic saline, HBSS or PBS, and then prepare a 0.5% suspension in HBSS or PBS.
3. Lymphocytes
   Suspend FH sedimented, adherence-depleted lymphocytes at a final concentration of approximately 5 x 10^6 lymphocytes/ml in the same medium used to prepare the SRBC.
4. Serum:
   For rosetting purposes, use heat-inactivated human AB serum, pooled serum from nontransfused male donors or fetal calf serum (FCS) found lacking cytotoxic activity and absorbed with SRBC as follows.
   a. Mix 1 volume of washed, packed SRBC with 2-3 volumes of serum.
   b. Spin the mixture to pellet cells and leave behind the absorbed supernatant.
   c. Use the absorbed serum immediately or freeze in aliquots, preferably at –70º C.
5. Fresh RPMI Or McCoy's Medium
6. Ficoll-Hypaque (FH)
7. Ammonium Chloride (NH_4Cl)
8. Petri Dish (Glass or Plastic)
9. Round Bottom Test Tube
10. Toluidine Blue

Instrumentation/Special Equipment

1. Refrigerated Centrifuge
2. 37º C incubator
3. Light Microscope
4. Hemacytometer
5. 4º C incubator

Calibration

Standard calibrations for centrifuge rotor speed, all thermometers and temperature regulated equipment, incubator percent CO_2, and microscopes should be performed and must be documented. Centrifuge and rotor should be capable of reaching appropriate speeds, generating appropriate g forces, and containing appropriate sized tubes.

Quality Control

1. Check viability of SRBC and lymphocytes daily.
2. Wash and resuspension media must be free from cytotoxic activity and contamination.
3. Do not use reagents that do not meet quality control criteria.
4. Record and maintain records of reagent and equipment quality control results.

Procedure

1. Isolate PBMC (see Lymphocyte Isolation Procedures).
2. Deplete the PBMC preparation of monocytes by adherence as follows:
   a. Adjust PBMC to a concentration of approximately 2-5 x 10^6 cells/ml in tissue culture media containing calcium and magnesium (e.g., RPMI-1640 + 10% non-cytotoxic pooled normal male human serum heat inactivated 30 minutes at 56º C).
   b. Pour the cell suspension into glass or plastic Petri dishes and incubate at 37º C for one hour.
   c. Following this incubation, briskly shake the plates to dislodge the nonadherent cells Pour off the nonadherent monocyte depleted fraction and save. This fraction usually contains 95% pure lymphocytes. A small number of lymphocytes will adhere to the plates as well during this process. This separation may be repeated to obtain a better population of lymphocytes, however, as many as five consecutive platings will not necessarily assure a 100% depletion of monocytes. For practical purposes, one, or at most two, platings are sufficient to remove most of the contaminating monocytes.
   d. An alternative method of monocyte removal from PBMC is by the use of carbonyl iron. However, this method is rather unreliable since some preparations are poorly phagocytized and carbonyl iron tends to adhere to a variety of cell types.[24]
   e. Evaluate the monocyte depletion. Determine the percent of remaining monocytes using a variety of tests such as ingestion of India ink or latex,[7] or histochemically by stain with nonspecific esterase.[30] The nonspecific esterase staining is one of the best procedures. However, it requires specialized reagents and procedures. Sometimes these procedures are available through a hematology laboratory.

3. Rosette T cells using the following steps:
   a. Coat a round-bottom tube with 0.1 ml of serum (see reagents) immediately prior to use.
   b. Combine 1 ml of lymphocyte suspension ($5x10^6$ cells/ml) to 1 ml of a 0.5% SRBC suspension in the serum-coated round bottom test tube.
   c. Mix the contents and centrifuge the tube 5 minutes at 200 x g. If more cells are needed for separation it is advisable to use a greater number of tubes.
   d. Incubate the pelleted cells overnight (18 hrs) at 4º C or, if time does not permit, for at least 2-3 hours before resuspension. If shorter incubations are desired, the use of one of the SRBC rosetting modifications provided later in this chapter is recommended.
   e. Following the overnight incubation, resuspend the SRBC lymphocyte pellet by gently rocking the test tube back and forth. Continue until a homogeneous cell suspension is achieved (i.e., dissipation of visible clumps).
4. Evaluate the rosettes as follows:
   a. Fill a hemacytometer with the suspension obtained in 3.e. above and view with a light microscope using the 45X objective.
   b. Score the number of rosette forming cells, i.e., those lymphocytes displaying three or more attached SRBC. To estimate viability, add a drop of toluidine blue before counting. Viable lymphocytes stain blue and may be seen as free cells or in the center of SRBC rosette, whereas dead lymphocytes appear purple.
5. Density gradient separation of E-rosette positive (T cells) from the E-rosette negative (B enriched) lymphoid cells.
   a. Carefully underlay the dispersed lymphocyte/SRBC suspension with 2 ml of FH solution.
   b. Centrifuge at 400 x g for 20 minutes at 4º C. Care should be taken to slowly increase the speed of the centrifuge and to insure that the test tubes are well balanced.
6. Due to their greater density, the E-rosetted T Cells will sediment at the bottom of the FH while the E-rosette negative cells, i.e., the B cell enriched fraction, will accumulate at the interface. The limitations for obtaining a fairly pure B cell preparation depend mainly on the efficiency of monocyte depletion and the formation and maintenance of the T cell E rosettes.
7. Recovery of T cells:
   a. Resuspend the rosettes in 1 ml of NH_4Cl for approximately 1-2 minutes, or until the turbidity disappears.
   b. Centrifuge and resuspend pelleted T cells in fresh RPMI, M^{c}Coy's, or other tissue culture medium.
   c. If red cells are still present, repeat NH_4Cl treatment for 2 minutes and resuspend in medium.

Calculations

Not applicable

Results

The use of fluoresceinated anti-immunoglobulin (Ig) reagents or immunofluorescence staining followed by analysis in the fluorescence-activated cell sorter (FACS) are two of the more commonly accepted methods for determining the extent of B cell purity when evaluating cell populations such as those obtained by E-rosetting.[11] Added discrimination between lymphocytes and monocytes can be achieved by using cells that have been exposed to latex ingestion prior to staining.[7, 20, 30] Latex-containing monocytes can be readily identified with phase contrast microscopy.

Procedure Notes

Using the above described rosetting method a maximum of 85% rosetting is expected. This percentage can be achieved provided that the correct reagents and optimal conditions, such as overnight incubation, even resuspension of pelleted cells and a careful underlayering on the Ficoll gradient, are met. Considering that 5-15% of the peripheral blood lymphocytes are B cells the assumption could be made that the remaining E-negative cells are mostly B cells provided that nearly 85% rosetting is obtained. However, under routine rosetting conditions, the percent rosetting with this method is not more than 60-80% at best and, consequently, the E-rosette negative population will be a mixture of mainly T and B cells plus monocytes. In order to improve the purity of B cells, re-rosette the E-negative population or alternatively use variants of the E-rosetting method which are designed to increase or stabilize these rosettes. A description of some of the more commonly used variations follows.

Limitations of Procedure

It is important to note that although the E-rosette test provides one of the best markers for human T cells, its use as a separation technique for isolating B cells has implicit limitations due to its indirect nature. Furthermore, even under the best experimental conditions, a large number of variables can influence the outcome of the rosette test.

1. SRBC
   Among the most frequent variables in E rosetting is the age of the SRBC. It is recommended that SRBC not be used after a storage of more than 2-3 weeks at 4ºC, although some batches continue to yield good results after 8-10 weeks of storage. Furthermore, differences may arise from one batch of SRBC to the next, although this should not lead to more than a 5-7% variability in the final rosetting.[27]

2. Lymphocyte viability
   The viability of the lymphocytes can also alter the results. Only the viable cells can rosette due to the need for an active metabolic process. Thus, unrosetted dead cells should be excluded in such calculations.
3. Rosettes
   a. If there is a delay in reading the percent of rosetted cells the slides should be kept at 4° C and for not more than 3-4 hours. Higher temperatures in particular will lead to the breakdown of rosettes.[11]
   b. When scoring rosettes it is important to identify the presence of the central lymphocyte in order to differentiate between an occasional red blood cell clump and the rosettes.
   c. Variations can arise due to the particular protein used in the assay, e.g., FCS, human serum or serum proteins such as BSA, which are all intended to stabilize the rosettes.[2, 11]
   d. In the rosetting technique the efficiency of separation seems to depend mainly on the rosette stability. Under most conditions, direct MoAb rosettes are distinctly more stable than indirect rosettes.
4. Procedural variations
   Procedural variations, such as centrifugation time, g-force, temperature or the length of time the cells are left in the lymphocyte/SRBC pellet,[8, 14] as well as the resuspension method, can all introduce differences.[21]
5. Health of the subject
   It is important to highlight the fact that while the normal percentage of E-rosettes for the human usually ranges between 60-85%,[26] various forms of disease, in particular those of immunological involvement, can alter these percentages drastically.[15, 18, 29]
6. Monocyte contamination
   Monocyte contamination is one of the most serious problems encountered in B cell isolation causing high background cytotoxicity in DR typing. Stux, et al.,[23] have found that the use of iodoacetamide (IAA) greatly alleviates this problem. Briefly, prior to DR typing, the separated B cells are resuspended at a concentration of 3.5×10^6 cells/ml in HBSS containing 0.01% IAA to increase membrane stability, and are then incubated for 30 minutes at RT.

Variations and Modifications of the E-Rosetting Method

A. Rosetting with Neuraminidase-Treated SRBC

Purpose

This modification of the E-rosetting procedure[1, 25] is probably the most widely used. Pretreatment of SRBC with neuraminidase tends to stabilize the rosettes and decrease their fragility. Neuraminidase seems to unmask the receptor sites on SRBC involved in the rosetting phenomenon while reducing the surface charge of these cells, thus enhancing their ability to more closely contact T cells.[9] Since the B cells do not participate in E-rosetting, the procedure can be viewed as a relatively good method for obtaining B cells in their natural state.

Specimen

Acceptable

Any whole lymphocyte population isolated by methods described in the lymphocyte isolation section of this manual.

Unacceptable

Whole lymphocyte populations which are less than 80% viable.

Reagents/Supplies

1. Labels
   All reagents must be properly labeled to indicate:
   a. Identity
   b. Titer, strength or concentration, when significant
   c. Preparation and/or expiration date
   d. Storage requirements, or other pertinent information
      Reagents must be stored according to manufacturers' instructions, at temperatures appropriate to maintaining its reactivity and specificity. Reagent performance must be checked before placing the reagent in service.
2. Phosphate-buffered saline (PBS)
3. Hanks' balanced salt solution (HBSS)
4. Neuraminidase
5. Ficoll Hypaque (FH)
6. NH_4Cl
7. Centrifuge tubes (50 ml and 10 x 75 mm)

Instrumentation/Special Equipment

1. Centrifuge and rotor capable of generating appropriate g forces and containing appropriate sized tubes.
2. 37º C incubator

Calibration

Standard calibrations for centrifuge rotor speed, all thermometers and temperature regulated equipment should be performed and must be documented

Quality Control

See Rosetting With Sheep Red Blood Cells in this chapter.

Procedure

1. Place 2 ml of commercially available, Alsever-maintained SRBC in a 50 ml centrifuge tube and dilute to 50 ml with PBS or medium and centrifuge 10 minutes at 400 x g.
2. Decant the supernatant and repeat the wash step 1-2 times until the supernatant shows no more signs of SRBC lysis.
3. Resuspend the remaining 0.5 ml SRBC pellet in 10 ml of HBSS or PBS to obtain a 5% SRBC suspension.
4. Add to this 0.2 ml of neuraminidase (Vibrio cholerae), specific activity 500 units/ml, and incubate the mixture at 37º C for 30 minutes.
5. Wash three times with HBSS or PBS and resuspend the remaining pelleted SRBC to a final 1% suspension. The SRBC must be freshly prepared daily. It is necessary to store neuraminidase in aliquots at 4º C.
6. Coat a 10 x 75 mm centrifuge tube with 0.1 ml of heat inactivated, absorbed serum.
7. Add 1 ml lymphocyte suspension (3-7 x 10^6 cells/ml) and 1% SRBC-N suspension to the coated tube.
8. Centrifuge at 200 x g for 10 minutes then allow the cells to incubate for 20 minutes at room temperature (RT).
9. Gently resuspend the rosettes in the manner previously described.
10. Underlay the suspended cell mixture with 1.5 ml of FH and continue the procedure as originally outlined above.
11. Recover the rosetted T cells, using the ammonium chloride treatment described previously.

Calculations

Not applicable

Procedure Notes

Although the percentage of rosetted cells by this modification is not necessarily higher than the one obtained by the original method, i.e., 60-80% rosetted cells, the modified method is preferable since it increases the durability and size of the rosettes, rendering them more suitable for further separation on the gradient. Thus, in the final analysis a B cell population less contaminated with T cells is obtained.

Limitations of Procedure:

See Rosetting With Sheep Red Blood Cells in this chapter.

B. Rosette Formation with AET-Treated SRBC

Purpose

This modification utilizes SRBC pretreated with the sulfhydryl compound 2-S-aminoethyl-isothiouronium bromide (AET). This method may be preferable since these rosettes form in less than one hour following the incubation of lymphocytes with the AET-SRBC. Furthermore, these rosettes are large, stable and resistant to mechanical disruption,[13, 19] and AET is also less expensive than neuraminidase.

Specimen

Acceptable

Any whole lymphocyte population isolated by methods described in the lymphocyte isolation section of this manual.

Unacceptable

Whole lymphocyte populations which are less than 80% viable.

Reagents/Supplies

1. Labels
   All reagents must be properly labeled to indicate:
   a. Identity
   b. Titer, strength, or concentration, when significant
   c. Preparation and/or expiration date
   d. Storage requirements, or other pertinent information
      Reagents must be stored according to manufacturers' instructions, at temperatures appropriate to maintaining its reactivity and specificity. Reagent performance must be checked before placing the reagent in service.
2. 2-S-aminoethyl-isothiouronium bromide (AET Solution)
   a. AET 402 mg
   b. distilled H_2O (d H_2O) 10 ml
   c. 4N sodium hydroxide (NaOH)
   d. Dissolve AET in d H_2O and adjust pH to 9.0 by dropwise addition of NaOH. Prepare fresh daily.
3. Phosphate-buffered saline (PBS)
4. RPMI
5. Fetal calf serum, heat-inactivated (FCS-HI)
6. Ficoll-Hypaque (FH)
7. Round bottom centrifuge tube (12 x 75 mm)
8. Crushed ice
9. NH_4Cl

Instrumentation/Special Equipment

1. Centrifuge
2. 37° C incubator
3. 4° C incubator
4. 37° C bath
5. Light microscope

Calibration

Standard calibrations for centrifuge rotor speed, all thermometers and temperature regulated equipment, incubator percent CO_2, and microscopes should be performed and must be documented. Centrifuge and rotor should be capable of reaching appropriate speeds, generating appropriate g forces, and containing appropriate sized tubes.

Quality Control

See Rosetting With Sheep Red Blood Cells in this chapter.

Procedure

1. Add 4 volumes of freshly prepared AET solution to washed, packed SRBC, and mix thoroughly.
2. Incubate the cells for 15 minutes at 37° C with frequent mixing.
3. Wash the SRBC-AET 5 times with cold PBS. Thoroughly resuspend the packed SRBC between centrifugations. (AET treatment tends to leave the cells somewhat sticky.)
4. Make the SRBC-AET up to 10% suspension in RPMI containing 20% SRBC absorbed, heat-inactivated FCS. The cell suspension can be used immediately or stored at 4° C for as long as five days.
5. Prepare 0.5% SRBC-AET suspension from the original 10% SRBC-AET suspension using RPMI-10% FCS as a diluent.
6. Add equal volumes of 0.5% SRBC-AET and lymphocyte suspensions (2 x 10^6 cells/ml) to a round bottom 12 x 75 mm test tube and mix.
7. Incubate the tube in a 37° C bath for 15 minutes and mix the cells frequently.
8. Centrifuge at RT at 200 x for 10 minutes.
9. Place the pelleted cells on crushed ice for a minimum of 45 minutes. Resuspend as usual, evaluate for rosetting under light microscope, and separate on FH. The percent of rosetting lymphocytes with this method is usually 65-85%, depending on the individual.
10. To recover T cells from these rosetted cells, the ammonium chloride treatment should be used, as previously described.

Calculations

Not applicable

Procedure Notes

See Rosetting With Sheep Red Blood Cells in this chapter.

Limitations of Procedure:

See Rosetting With Sheep Red Blood Cells in this chapter.

C. Rosetting in the Presence of Dextran

Purpose

The observation has been made that Dextran, added to the lymphocyte-SRBC mixture at optimal concentrations, can serve to enhance rosette formation. This may be due, in part, to a reduction in the electric charge at the cell surface which, in turn, allows better cell to cell contact.[4]

Specimen

Acceptable

Any whole lymphocyte population isolated by methods described in the lymphocyte isolation section of this manual.

Unacceptable

Whole lymphocyte populations which are less than 80% viable.

Reagents/Supplies

1. Labels:
   All reagents must be properly labeled to indicate:
   a. Identity
   b. Titer, strength, or concentration, when significant
   c. Preparation and/or expiration date
   d. Storage requirements, or other pertinent information
2. Hanks' balanced salt solution (HBSS)
3. Phosphate-buffered saline (PBS)
4. Dextran (average MW of 70,000 daltons)
5. Ice bath

Instrumentation/Special Equipment:

Centrifuge and rotor capable of reaching generating appropriate g forces, and containing appropriate sized tubes.

Calibration

Standard calibrations for centrifuge rotor speed, all thermometers and temperature regulated equipment and microscopes should be performed and must be documented.

Quality Control

1. Check viability of SRBC and lymphocytes daily.
2. Wash and resuspension media must be free from cytotoxic activity and contamination.
3. Reagents must be stored according to manufacturers' instructions, at temperatures appropriate to maintaining its reactivity and specificity.
4. Reagent performance must be checked before placing the reagent in service. Do not use reagents which do not meet quality control criteria.
5. Record and maintain records of quality control results.

Procedure

1. Prepare suspensions of SRBC (0.5%) and lymphocytes in HBSS or PBS, pH 7.0 with 0.1% BSA.
2. Mix equal volumes of the cell suspensions and incubate 20 minutes at 30° C in the presence of 4-7% Dextran.
3. Centrifuge the cell mixture 5 minutes at 200 x g and place on ice for 1 hour.
4. Resuspend the cells gently and proceed as described above for the separation of E-positive and E-negative populations.

Calculations

Not applicable

Procedure Notes

See Rosetting With Sheep Red Blood Cells in this chapter.

Limitations of Procedure

See Rosetting With Sheep Red Blood Cells in this chapter.

D. Rosetting with Anti-Human-Fab-Coated SRBC

Purpose

The direct method of B cell separation is based on the presumption that anti-human-Fab-coupled SRBC will bind to such modified SRBC.[18] The rosetted population can be separated from the non-rosetted population by the usual FH separation. Recovery of the rosette positive B cell population is accomplished by lysis of the B cell-attached SRBC through hypotonic shock with distilled water or ammonium chloride.

Specimen

Acceptable

Any whole lymphocyte population isolated by methods described in the lymphocyte isolation section of this manual.

Unacceptable

Whole lymphocyte populations which are less than 80% viable.

Reagents/Supplies

1. Labels:
   a. All reagents must be properly labeled to indicate:
   b. Identity
   c. Titer, strength, or concentration, when significant
   d. Preparation and/or expiration date
   e. Storage requirements, or other pertinent information
2. $F(ab')_2$ anti-human-Fab (Cappel Laboratories)
3. Saline solution of chromium chloride ($CrCl_3$)
4. Ammonium chloride (NH_4Cl)
5. Ficoll-Hypaque (FH)

Instrumentation/Special Equipment

1. Centrifuge and rotor with capability of generating appropriate g forces, and containing appropriate sized tubes.
2. Microscope

Calibration

Standard calibrations for centrifuge rotor speed, all thermometers and temperature regulated equipment and microscopes should be performed and must be documented

Quality Control

See Rosetting With Sheep Red Blood Cells in this chapter.

Procedure

1. To 0.5ml of washed packed SRBC, add, in order, 0.5ml of each of the following:
   a. 1% saline solution of $CrCl_3$
   b. Affinity chromatography purified rabbit, $F(ab')_2$ anti-human-Fab (1 mg/ml saline)
2. Incubate 5 minutes at RT.
3. Wash 3 times in excess saline.

4. Resuspend the cells in saline to a final volume of 15 ml. The antibody-coated cells can be kept up to 10 days at 4º C.
5. Wash the lymphocytes with saline to remove all immunoglobulin from non-B cells and adjust concentration to 5 x 10^6 cells/ml.
6. Mix equal volumes of lymphocyte and antibody-coated SRBC suspensions and centrifuge 5 minutes at 200 x g.
7. Vigorously resuspend the pellet with a pipette.
8. Examine the suspension under microscope to determine percent rosetting.
9. Isolate B cells as follows:
   a. Place the cell suspension on FH gradient to separate rosette positive and negative cells. The rosette positive, B enriched fraction will sediment at the bottom of the tube.
   b. Discard the supernatant and resuspend the sedimented cells in cold d H_2O or cold 0.17M NH_4Cl for 2-5 minutes to lyse the SRBC.
   c. Immediately resuspend the cells in excess medium and wash 3 times to remove remaining red cell stroma and restore proper tonicity.

Calculations

Not applicable

Procedure Notes

See Rosetting With Sheep Red Blood Cells in this chapter.

Limitations of Procedure

In terms of the limitations of this method, one has to consider the fact that Ig positive cells, whether displaying intrinsic membrane Ig or absorbed Ig, will tend to rosette. Thus, the precautions described earlier with respect to evaluating percentages of B cells by anti-Ig staining will apply here as well. When used properly this approach is an expedient and relatively simple method for obtaining greater than 90% pure B cells.

E. Rosetting with Ox RBC and Monoclonal Antibody

Purpose:

The direct monoclonal antibody (MoAb) rosetting technique described here is a simple, reliable and very sensitive method of isolating B cells. B cells will attach to the H4 MoAb coupled to chromium chloride treated Ox erythrocytes (OxE) and form rosettes leaving the T lymphocytes free. The T lymphocytes can be centrifuged out by using a density gradient which leaves the rosetted B cells at the bottom of the tube and the T cells in the interface. The B cells are released by using ammonium chloride lysis.

Specimen

Acceptable

Any whole lymphocyte population isolated by methods described in lymphocyte isolation section of this manual.
Note: Up to 15 x 10^6 lymphocytes can be rosetted per 1 ml of H4-Ox-E Solution.

Unacceptable

Whole lymphocyte populations which are less than 80% viable.

Reagents

1. Labels:
   All reagents must be properly labeled to indicate:
   a. Identity
   b. Titer, strength, or concentration, when significant
   c. Preparation and/or expiration date
   d. Storage requirements, or other pertinent information
   Reagents must be stored according to manufacturers' instructions, at temperatures appropriate to maintaining its reactivity and specificity. Reagent performance must be checked before placing the reagent in service.
2. Ox RBC Suspension
   a. Mix the bottle of OxE well and extract desired amount (20 ml unwashed equals 5 ml packed RBC).
   b. Wash the RBCs 5X in 0.85% isotonic sodium chloride as follows: Spin the ox blood for 2 minutes at 3000 x g. Discard the supernatant. Be careful to remove all of the white buffy coat. Add saline, mix well, and repeat.

   c. After the 5th wash, packed cells are ready for coupling.
      Note: The above reagent should be prepared the day of coupling.
3. Chromium Chloride ($CrCl_3$) Coupling Reagent Preparation
   a. $CrCl_3$ 20 mg
      NaCl solution 50 ml
   b. Dissolve $CrCl_3$ in NaCl solution. Store 1 week before using. This solution will keep indefinitely at 4° C.
4. Ammonium Chloride (NH_4Cl)
   a. Sterile, distilled H_2O 1.0 L
      NH_4Cl 7.5 g
      Tris-HCl buffer 1.0 g
   b. Dissolve NH_4Cl and Tris-HCl in water. Adjust pH to 7.2. Keep refrigerated. Shelf life is approximately 6 months.
5. Tris-hydrochloric acid (HCl) Buffer.
6. Percoll Stock Solution
   1 part of 10X PBS and 9 parts of Percoll (1:10). The 72% Percoll, like the $CrCl_3$ solution, will vary with each batch. Each new batch of Percoll should be tested to make certain that no rosetted cells remain in the interface after centrifugation. If rosettes remain in the interface, a new batch of Percoll should be prepared and tested.[21]
7. 10X PBS Solution
   1.37M sodium chloride (NaCl) 16.0 g
   0.027M potassium chloride (KCl) 4.0 g
   0.081M sodium phosphate (Na_2HPO_4) 2.3 g
   0.015M potassium phosphate (KH_2PO_4) 4.0 g
8. MoAb H4 (Anti-Ia) (UCLA Tissue Typing Laboratory)
9. Hanks' balanced salt solution (HBSS)
10. McCoy's

Instrumentation/Special Equipment:

1. Fisher Microcentrifuge

Calibration:

Standard calibrations for centrifuge rotor speed, all thermometers and temperature regulated equipment, incubator percent CO_2, and microscopes should be performed and must be documented. Centrifuge and rotor should be capable of reaching appropriate speeds, generating appropriate g forces, and containing appropriate sized tubes.

Quality Control:

See Rosetting With Sheep Red Blood Cells in this chapter.

Procedure

1. Prepare MoAb coupled OxE as follows. This protocol is for the preparation of 10 ml coupled RBC (enough to isolate B cells from 10 samples).
   a. Prepare saline washed OxE pellet according to instructions in reagent preparation.
   b. Add 0.1 ml of the MoAb H4 to 0.1 ml aliquot of the OxE pellet and mix well.
   c. Add 0.2 ml $CrCl_3$ to the H4-OxE mixture and mix thoroughly.
   d. Incubate at RT for 30 minutes mixing gently at 10 minute intervals.
   e. Wash the OxE four times in HBSS or similar medium.
   f. Resuspend the coupled OxE in 10 ml McCoy's medium with 0.5% heat inactivated FCS. This solution is a 1% coupled OxE solution.
   g. Refrigerate the coupled OxE when not in use. The refrigerated shelf life is approximately 7-10 days. Up to 15 x 10^6 lymphocytes can be rosetted with 1 ml of the H4-OxE solution.
2. Isolate a whole lymphocyte population by a method of your choice.
3. Rosette B cells as follows:
   a. Centrifuge to pellet 3-15 x 10^6 lymphocytes in a Fisher tube. Decant supernatant completely and discard.
   b. Resuspend the pellet in 1 ml of H4 coupled OxE and centrifuge at 500 x g for 5 minutes. Let the cells sit at room temperature for 10 minutes. Decant supernatant completely and discard.

4. Isolate the B cells as follows:
   a. Resuspend the pellet gently, but completely, in 1 ml of 72% Percoll and divide equally into 2 Fisher tubes.
   Note: Pellet must be resuspended completely so that no visible clumps remain.
   b. Layer HBSS or PBS over the 0.5 ml 72% Percoll suspended cells in Fisher tubes. Spin for 2 minutes at 1500 x g. The interface contains T cells, and the pellet contains rosetted DR positive cells.
   c. Depending upon need, save or discard the interface and supernatant, being careful not to contaminate interface cells with rosetted cells in the pellet.
   d. Resuspend the pellets in 1 ml of NH_4Cl until turbidity disappears (usually 2 minutes or less). Spin at 1000 x g for 1 minute. If red cells are still present, repeat NH_4Cl treatment for 2 minutes.
   Note: Lysis with ammonium chloride must be complete.
   e. Wash twice, count, and adjust to 1.5-2.0 x 10^6 cells/ml for addition to typing trays. Studies have indicated that a higher percentage of diseased patients' B cells will react if the cells are incubated at 37° C before adding them to the trays. Therefore B cells should be incubated for 2 hours at 37° C after the final adjustment before plating.

Calculations

Not applicable

Results

In the normal lymphocyte population 80% of the lymphocytes are T cells and 20% are B cells. One would expect that if more than 20% yield is retrieved at the end of this procedure from the total starting lymphocyte count, there may be T cell contamination.

Procedure Notes

1. Each batch of $CrCl_3$ will differ in its ability to couple. Therefore it is important to test each new batch against a solution which works well, prior to placing the new batch in use.
2. It has been reported that B cells separated with this method are more pure and can improve the frequency of successful DR typing in dialysis patients to more than 90%.
3. Wilhelm, et al.[26] have also reported success in isolating T cells and monocytes using appropriate discriminative monoclonal anti-leukocyte antibodies directly coupled to OxE. These techniques depend upon the availability of good selective MoAb and the minimal amounts of MoAb needed for optimal coating of OxE must be empirically determined.
4. The H4MoAb used to isolate B cells in this procedure is available commercially, and the optimal amount needed for OxE coating has been predetermined by the manufacturer.

Limitations of Procedure

See Rosetting With Sheep Red Blood Cells in this chapter.

References

1. Bentwich Z, Douglas SD, Skutelsky E and Kunkel HG, Sheep red cell binding to human lymphocytes treated with neuraminidase; enhancement of T cell binding and identification of a subpopulation of B cells. J Exp Med 137:1532, 1973.
2. Bentwich Z, Douglas SD, Siefal FP and Kunkel HG, Human lymphocyte-sheep erythrocyte rosette formation: Some characteristics of the interaction. Clin Immunol Immunopathol 1:511, 1973.
3. Boyum A, Isolation of mononuclear cells by one centrifugation, and of granulocytes by combining centrifugation and sedimentation at 1 g. Scand J Clin Lab Invest 21 Suppl 97:77, 1968.
4. Brown CS, Halpern H and Wortis HH, Enhanced rosetting of sheep erythrocytes by human peripheral blood T cells in the presence of Dextran. Clin Exp Immunol 20:505, 1975.
5. Chess L, MacDermott RP and Schlossman SF, Immunologic functions of isolated human lymphocytes subpopulations. I. Quantitative isolation of human T and B cells and response to mitogens. J Immunol 113:1113, 1974.
6. Cicciarelli JC, Ayoub G, Terasaki PI and Billing R, Improved HLA-DR typing of dialysis patients using monoclonal antibodies. Transplantation 33:558, 1982.
7. Cline MJ, and Lehrer RI, Phagocytosis by human monocytes. Blood 32:423, 1968.
8. Froland SS, Binding of sheep erythrocytes to human lymphocytes: A probable marker of T lymphocytes. Scand J Immunol 1:269, 1972.
9. Galili U, and Schlesinger M, The formation of stable E rosettes after neuraminidase treatment of either human peripheral blood lymphocytes or of sheep red blood cells. J Immunol 112:1628, 1974.
10. Howard FD, Ledbetter JA, Wong J, Bieber CP, Stinson EB and Herzenberg LA, A human T lymphocyte differentiation marker defined by monoclonal antibodies that block E rosette formation. J Immunol 126:2117, 1981
11. Jondal M, Holm G, and Wigzell H, Surface markers on human T and B lymphocytes. I. A large population of lymphocytes forming nonimmune rosettes with sheep red blood cells. J Exp Med 136:207, 1972.

12. Kamoun M, Kadin ME, Martin PH, Nettleton J and Hansen JA, A novel T cell antigen preferentially expressed on mature T cells and shared by both well and poorly differentiated B cell leukemias and lymphomas. J. Immunol 127:987, 1981.
13. Kaplan MR and Clark C, An improved rosetting assay for detection of human T lymphocytes. J Immunol Methods 5:131, 1974.
14. Lay WH, Mendes NF, Bianco C and Nussenzweig V, Binding of sheep red blood cells to a large population of human lymphocytes. Nature (London) 230:531, 1971.
15. Lisak RP, Levinson AI, Zweiman B and Abdou NI, T and B lymphocytes in multiple sclerosis. Clin Exp Immunol 22:30, 1975.
16. Loon J and Takemura S, Personal communication. One Lambda, Inc. 1988.
17. Mendes NF, Tolnai MEA, Silveira NPA, Gilbertsen RB and Metzgar RS, Technical aspects of the rosette tests used to detect human complement receptor (B) and sheep erythrocyte-binding (T) lymphocytes. J Immunol 111:860,1973.
18. Messner RP, Lindstrom FD and Williams RC, Peripheral blood lymphocyte cell surface markers during the course of systemic lupus erythematosus. J Clin Inv 52:3046, 1973.
19. Pellegrino MA, Ferrone S, Dierich MP and Reisfeld RA, Enhancement of sheep red blood cell human lymphocyte rosette formation by the sulfhydryl compound 2-amino ethyl-isothiouronium bromide. Clin Immunol Immunopathol 3:324, 1975.
20. Preud'homme JL and Flandrin G, Identification by peroxidase staining of monocytes in surface immunofluorescence tests. J Immunol 113:1650, 1974.
21. Steele CM, Evans J and Smith MA, The sheep-cell rosette test on human peripheral blood lymphocytes: An analysis of some variable factors in the technique. Br J Haematol 28:245, 1974.
22. Stux S, Dubey D and Yunis E, Rosetting as a method of separating Human B cells. In: AACHT Laboratory Manual; AA Zachary and WE Braun, eds.; American Society for Histocompatibility and Immunogenetics New York; I-7-1, 1981.
23. Stux S, Hammond P, Fitzpatrick D, Dubey D, and Yunis E, Use of monocytes in HLA-A,B,C, and DR typings. Tissue Antigens 15:152, 1980.
24. Tebbi K, Purification of lymphocytes. Lancet 1:1392, 1973.
25. Weiner MS, Bianco C, and Nussenzweig V, Enhanced binding of neuraminidase-treated sheep erythrocytes to human T lymphocytes. Blood 42:939, 1973.
26. Wilhelm M, Pechumer H, Rank G, Kopp E, Reithmuller G and Rieber EP. Direct monoclonal antibody rosetting. J Immunol Methods 90:89, 1986.
27. Wortis HH, Cooper AG and Brown MC, Inhibition of human lymphocyte rosetting by anti-T sera. Nature (London). New Biol 243:109,1973.
28. Wybran J, Carr MC, and Fudenberg HH, The human rosette-forming cell as a marker of a population of thymus-derived cells. J Clin Invest 51:2537,1972.
29. Wybran J and Fudenberg HH, Thymus-derived rosette-forming cells in various human disease states: Cancer, lymphoma, bacterial and viral infections, and other diseases. J Clin Invest 52:1026, 1973.
30. Yam LT, Li CY and Crosby WH, Cytochemical identification of monocytes and granulocytes. Am J Clin Pathol 55:283, 1971.

Isolation of Monocytes from Peripheral Blood Mononuclear Cells

Myra Coppage

Purpose

Mononuclear cells obtained from peripheral blood by flotation on Ficoll-Hypaque contain mainly T lymphocytes, B lymphocytes and *monocytes.* The property of strong adherence of monocytes to plastic surfaces facilitates their separation from lymphocytes. The purity of the monocyte population depends on efficient removal of these non-adherent cells. Monocytes express HLA-A,B,C and DR antigens but DQ is present in low amounts or is not detectable in more than half of the resting normal monocytes from human peripheral blood. Non-HLA alloantigens are also present on monocytes. It should be remembered that the plastic *adherence* method for isolating monocytes may also activate cells (upregulate gene expression) which may affect assays in which the cells are used.

Specimen

Peripheral blood collected into sodium heparin or ACD. Monocyte isolation should be performed within 24 hours for maximum yield.

Reagents and Supplies

1. Heparin Sodium or ACD
2. Hanks' Balanced Salt Solution (HBSS)
3. RPMI Medium 1640
4. Ficoll-Hypaque (FH) solution (density 1.077)
5. graduated pipets; 5 and 10 ml
6. Pooled Human Serum (PHS). Pool of normal male untransfused donors, sterilized by filtration, heat inactivated and tested to ensure it is not toxic
7. Phosphate Buffered Saline (PBS)
8. 0.02% EDTA (disodium ethylenediamine tetraacetate) in PBS
9. 15 ml polypropylene, conical tubes
10. 25x100 tissue culture grade dishes, or 75 cm^2 tissue culture flask
11. Acridine orange (100 µg/ml; SIGMA) in PBS

Instrumentation

1. Beckman GS centrifuge with GH 3.7 horizontal rotor (or equivalent)
2. 37° C, 5% CO_2 humidified incubator
3. Fluorescence microscope

Calibration

Standard calibrations for centrifuge rotor speed, all thermometers and temperature regulated equipment, incubator percent CO_2, and microscopes should be performed and must be documented. Centrifuge and rotor should be capable of reaching appropriate speeds, generating appropriate g forces, and containing appropriate sized tubes.

Quality Control

1. It is critical that tissue grade petri dishes and flasks be tested for appropriate adherence properties prior to use.
2. Standard reagent and equipment QC procedures should be performed and must be documented.

Procedure

1. Dilute anticoagulated, peripheral blood with an equal volume of HBSS and place 10 ml volumes in 15 ml conical tubes.
2. Carefully layer the FH solution under the blood/PBS taking care to maintain an interface.
3. Centrifuge at 2000 rpm (900 x g) for 30 min at room temperature (RT) using no brake.

4. Aspirate mononuclear cells from the interface and wash twice with RPMI 1640 medium. Resuspend in RPMI 1640 medium containing 10% pooled human serum.
5. Adhere monocytes by culturing 20-30 x 10^6 mononuclear cells in 25 ml of RPMI 1640 medium with 10% human serum in two or three 10 cm diameter plastic dishes of tissue culture quality. Incubate at 37° C in a humid atmosphere containing 5% CO_2 for at least one hour. The cultures may be held at this step overnight.
6. Aspirate the non-adherent cells (lymphocytes) with a pipet. Wash the dishes thoroughly (4-6 times) with a stream of RPMI with serum at 37° C with a 5 or 10 ml pipet to ensure that all non-adherent cells are removed. Check for completeness by inspection with an inverted phase-contrast microscope.
7. When only adherent cells remain; release the monocytes by adding 3 ml cold 0.02% EDTA in PBS for 10 min and gently tapping the dishes or flask. Check for their release under the scope.
8. Add 9 ml RPMI with 10% serum to the dishes and transfer to a 15 ml conical tube and centrifuge for 10 minutes at 1400 rpm (400 x g). Resuspend pellet in 2 ml RPMI 1640 with 10% serum.
9. Determine concentration and viability by staining with trypan blue. For determination of monocyte purity, adjust to approximately 1 x 10^6 and stain with acridine orange. Add 1 µl of acridine orange solution to 25 µl cell suspension and place one drop on a microscope slide and cover. Observe under fluorescence microscope using a 40-60X objective.
10. Acridine orange intercalates DNA and will stain the nucleus green. It also binds to RNA, but stains red. Viable lymphocytes will appear almost completely green while monocytes will demonstrate a bean shaped nucleus fluorescing green and a larger cytoplasmic component which stains red.

Calculations

To determine monocyte purity, count one hundred cells. The number of monocytes/100 x 100 yields the percent of monocytes obtained.

Results

Expect to obtain 1.5-3 x 10^6 monocytes from 30 ml of blood with a viability and purity of 80% or better.

Procedure Notes

Troubleshooting

1. Few adherent cells obtained.
2. The most common cause of this problem is poor adherence of monocytes due to the use of inadequate plastic dishes. It is important to use tissue culture quality dishes or a tissue culture flask. In addition, the number of monocytes in peripheral blood of individuals varies, and may be effected by medication and disease status.
3. Low viability of monocytes.
4. Make sure that, after adherence, the monocytes are collected in medium containing serum. The pooled human serum must be pretested to determine that it is not toxic for monocytes.
5. Purity of cell preparations not satisfactory.
6. Contamination by non-adherent cells is probable and is the result of insufficient washing after adherence.

Common Varations

1. Isolation by size sedimentation.
   Monocytes have a specific density which allows them to be collected specifically using a Percoll (Pharmacia) gradient (specific gravity 1.130 g/ml).
2. Isolation by immunomagnetic beads.
   Magnetically charged, polystyrene beads are available and may be coated with monocyte specific monoclonal antibody. When incubated with the beads (in the cold) the beads will rosette with the cells and the cells isolated by placing the tube with the cell suspension against a magnet. For most applications the beads should be detached as monocytes are phagocytic cells and will incorporate the beads.
3. Isolation by flow cytometry
   Alternatively, monocytes can be isolated by sorting via flow cytometry. Briefly cells are labeled with a monocyte specific fluorescent tag (Leu M3 or M5; Becton Dickinson) which allows the tagged cells to be separated from the population. These tags may also be used in the assessment of purity of other isolation methods.

Limitations of Procedures:

1. Monocytes die off quickly when improper anticoagulants are used.
2. Tissue culture grade dishes and flasks are critical to this isolation procedure.

References

1. Cerilli J, Brasile L, Clarke J, and Galouzis T, The vascular endothelial cell-specific antigen system: Three years experience in monocyte crossmatching. Transplant Proc 17:567, 1985.
2. Colbaugh P, and Stastny P, Antigens in human monocytes. III. Use of monocytes in typing for HLA-D related (DR) antigens. Transplant Proc 10:871, 1978.
3. Wahl LM and Smith PD, Isolation of monocyte/macrophage populations. In: Current Protocols in Immunology, Coico R, series ed., John Wiley and Sons, USA; Inc Vol 2, Section 7.6.1, 1994.

Isolation of Endothelial Cells

Nufatt Leong

Purpose

A unique tissue-specific *endothelial cell* antigen system was reported independently by Stastny, Cerilli, and Paul in the 1970's. The endothelium, which lies as a barrier between the circulating blood and the vascular walls, plays a key role in (1) maintaining normal hemostasis, (2) in influencing vascular permeability, (3) phagocytosis, and (4) antigen presentation. Given the numerous functions of the *vascular endothelial cells (VEC)*, it is one may postulate a possible role in allograft rejection. Indeed, Cerilli[3] presented evidence of specific anti-VEC alloantibodies in HLA identical living related renal transplants. Based on these findings, the screening of anti-VEC may be desirable in cases where rejection of graft occurred with no detectable anti-HLA antibodies.

Endothelial cells can be harvested from aorta, skin, adipose tissue, and cornea, however, the cell yields are too low for testing. The best source of VEC is the intimal lining of umbilical cord vein where higher cell yield can be obtained easily by enzymatic digestion.

Specimen

Umbilical cord not more than 48 hrs old.

Reagents and Supplies

1. Umbilical cord tape
2. 3 way stopcock
3. 20cc syringe
4. Trypsin-EDTA solution (Gibco)
5. Collagenase (cls I, Worthington)
6. M199 Medium (Gibco), supplemented with 10 mM HEPES, 0.1mg/ml Gentamicin, 2mM L-Glutamine.
7. 1M HEPES buffer (Gibco)
8. Gentamycin (Gibco)
9. L-Glutamine (Gibco)
10. Characterized Fetal Bovine Serum (Hyclone)
11. Endothelial cell growth factor (SIGMA; 100X)
12. Formalin (40%)
13. Triton X 100
14. Goat anti human factor VIII (ICN), use at 1:50 diluted in PBS
15. FITC conjugated rabbit anti goat IgG (ICN), use at 1:30 diluted in PBS
16. Phosphate buffered saline (PBS)
17. Chambered tissue culture slides
18. Fluoromount G

Instrmentation/Special Equipment

1. 37° C waterbath
2. Centrifuge
3. Incubator, humidified at 37° C with 5% CO_2
4. Fluorescence microscope
5. Phase contrast microscope

Calibration

1. Standard calibrations for centrifuge rotor speed, incubator, temperature and percent CO_2 and microscopes should be performed and must be documented.

Quality Control

1. It is critical that tissue grade petri dishes and flasks be tested for appropriate adherence properties prior to use.
2. Standard reagent and equipment QC procedures should be performed and must be documented. In particular, refrigerator thermometers must be verified.

Procedure

1. Wash the outside of the umbilical cord with PBS with 0.1 mg/ml of Gentamicin added.
2. Check visually to make sure there are no punctured site(s) on the cord.
3. Make even cuts at both ends of the umbilical cord.
4. Identify the cord vein and insert a 3-way stopcock into each end; tie securely with umbilical tape.
5. Flush the vein with PBS until no blood is visible.
6. Close the stopcock at one end, then fill the vein with collagenase (0.75 mg/ml in PBS) from the other end, close the stopcock.
7. Incubate in 37° C incubator for 10 min.
8. Open both ends, rinse the vein with M199 + 10% FBS into 15 ml conical tubes; use at least twice the original volume of the collagenase solution.
9. Pellet the cells in centrifuge at 1200 rpm, room temperature, for 10min.
10. Decant supernatant, resuspend in 5 ml of M199 (supplemented with 5 µl/ml growth factor and 20% FBS), and transfer to a 25 cm^2 tissue culture flask (Corning).
11. Place the flask in a humidified incubator at 37° C with 5% CO_2 overnight.
12. Aspirate media with a 5 ml pipet, rinse twice with warm M199, add 5 ml fresh M199 with additives as in 10.; incubate until confluent (24 to 96 hrs).
13. To detach the adherent VEC from flask, remove media and rinse twice with 5 ml volumes of Calcium and Magnesium free PBS.
14. Add 1 ml of Trypsin-EDTA and let stand at room temperature for 1-3 min, then tap the flask against the palm of the hand. Add 10 ml of M199 (with 10% FBS) and flush the surface where the cells adhered. Transfer to a 15 ml centrifuge tube, and pellet cells at 1200 rpm for 10 min.
15. Decant supernatant and resuspend cells in M199 with 10% FBS. Count in hemoctyometer and adjust to desired concentration with media. Determine purity by factor VIII staining as below.

Factor VIII Staining

1. Add one drop of VEC from Step #15 to a chambered slide, fill with media and incubate overnight.
2. Wash 3x with PBS with 5% FBS (PBS-5).
3. After final wash remove PBS-5 and add 2 drops of 40% Formalin. Fill chamber with PBS-5, let stand at room temperature for 10-15min.
4. Wash 3x with PBS-5. Add 250 µL of 0.5% Triton X-100, let stand at room temperature for 15 min.
5. Wash 3x with PBS-5. Add 250 µL of goat anti-human factor VIII (1:50), incubate one hour at room temperature.
6. Wash 3x with PBS-5. Add 200 µl of FITC conjugated rabbit anti-goat IgG (1:30), incubate one hour at room temperature.
7. Wash 3x with PBS-5, then 3x with distilled water.
8. Remove chamber and shake off excess water. Add one drop of Fluromount G to each well.
9. Put coverslip on slide, blot off excess Fluromount G, and view under fluorescence microscope.

Results

Generally, greater than 1x 10^6 cells can be obtained from each cord. The viability and purity of VEC should be greater than 90% as determined by trypan blue exclusion and factor VIII staining. Usually, two days culture is sufficient for confluency, however, culture up to one week may be required. Poor viability after trypsin digestion can be overcome by using Percoll density gradient to separate out dead cells.

Procedure Notes

1. Bacterial contamination can be a hazard. Strict sterile technique must be employed during the isolation procedure.
2. The digestion steps with typsin and collagenase should not be extended as prolonged exposure to these enzymes can begin to damage the endothelial cells.

Limitations of Procedure

1. Not enough cells.
   The cord is too short or collagenase solution is old. Start with a longer cord and fresh preparation of collagenase.
2. Poor viability.
   The tissue is too old after delivery. Make sure the cord is no more than 48 hrs old.

References

1. Cerilli J et al., The significance of antivascular endothelial cell antibody-its role in transplantation. Surg Gynecol Obstet 135:246, 1972.
2. Cerilli J et al., Role of antivascular endothelial antibody in predicting renal allograft rejection. Transplant Proc 9:771, 1977.
3. Cerilli J et al., The Vascular Endothelial Cell antigen system. Transplantation 39(3):286-289, 1985.
4. Gimbrone MA, Culture of Vascular Endothelium. In: *Progress in Hemostasis and Thrombosis*, Spacet TH ed., Grune and Stretton, NY, pp.1- 28, 1976.
5. Jaffe EA et al., Culture of human endothelial cells derived from umbilical cord vein. J Clin Invest 51:46a, 1972.
6. Moraes JR, and Stastny P, A new antigen system expressed in human endothelial cells. J Clin Invest 60:449, 1977.
7. Paul LC et al., Vascular Endothelial Alloantigens in renal Transplantation. Transplantation 40(2): 117-123, 1985.

Isolation of Granulocytes

Prema R. Madyastha

Purpose

Cell-surface alloantigens specific to human granulocytes have been identified during the past two decades. These antigens, designated as human granulocyte or neutrophil specific antigens, under incompatible or pathological conditions stimulate granulocyte specific antibodies. The resulting antigen-antibody reactions cause alloimmune neutropenias as well as primary or secondary autoimmune neutropenias, and febrile and pulmonary transfusion reactions.[3, 7] Thus, pure granulocytes are important to detect and characterize antibodies[5] that react with antigens specific to granulocytes. This chapter describes a granulocyte-isolation procedure suitable for this purpose.

Human blood cells have different densities, and utilizing this property and a Ficoll-Hypaque double density gradient (a lighter gradient (LG) with a specific gravity of 1.08 and a heavier gradient (HG) with a specific gravity of 1.12), pure granulocytes may be obtained that are free from lymphocytes, platelets and red cells.[1, 2, 4, 6] Ficoll is a hydrophilic sucrose polymer of high molecular weight (mol wt 400,000) that must be dehydrated prior to gradient preparation. Ficoll solutions are highly viscous, but with the addition of Hypaque (sodium diatrizoate) specific densities can be prepared that are very suitable for cell separation.[1] This procedure contains three steps:

1. Removing the bulk of the red cells using methylcellulose-15 which is a non-toxic chemical widely used as an erythrocyte sedimenting agent. First methylcellulose is mixed in an appropriate proportion with the blood. Then the blood is either kept at room temperature for 30-45 min for gravity sedimentation or lightly centrifuged for 7-10 min at 30g to remove the bulk of the red cells. Methylcellulose selectively reacts with the red cells and forms aggregates. This facilitates faster sedimentation of RBCs, leaving all the white cells in the supernatant plasma.
2. This leukocyte rich plasma with very few contaminated red cells is then layered on the double density gradients to further separate granulocytes. After centrifugation , lymphocytes and platelets form the first layer on top of the lighter gradient and the granulocytes form a second layer at the interface of the two gradients. The contaminated red cells form a button at the bottom of the tube.
3. Finally, the pure granulocytes are aspirated, washed, and in appropriate concentrations, are directly employed in granulocyte agglutination and immunofluorescent techniques to detect granulocyte antibodies. The granulocytes are free of red cells and do not require the hypotonic lysis step to remove red cells. Thus prepared, the viability of granulocytes is extremely good, which makes it particularly suitable for granulocytotoxicity techniques.

Specimen

Acceptable Specimen

10-20 cc of whole blood collected in tubes containing ethylenediaminetetraacetic acid, (EDTA-Na_3) as anti-coagulant. The specimen should be collected, stored and received at room temperature and should be processed within 18 hours of collection . The isolation of granulocytes should begin as soon as they are received in the lab and the isolated cells should be employed immediately in the assays..

Unacceptable Specimen

Blood obtained: without anticoagulant (clotted)
in other types of anti-coagulant (loss of granulocyte yield),
received frozen or stored at 18° C or less (non-specific) or
received more than 18 hours of collection (hemolyzed).

Reagents and Supplies:

Supplies

1. Glass beaker (1 liter volume) (to prepare 1% methyl cellulose, 9% Ficoll and EDTA buffer) – 3
2. Glass beaker (500 ml volume) (to prepare 0.9% physiological saline, Ficoll-Hypaque-1.08; and Ficoll-Hypaque-1.12) – 3
3. Volumetric flasks (500 ml volume) – 1; (100 ml volume) – 1
4. Magnetic stirrer and stir bars
5. Mettler balance and weighing cups or wax coated-paper
6. Measuring cylinder (100 ml volume) – 1
7. Plastic conical centrifuge tubes (50 ml volume) 40 (for storing methyl cellulose and Ficoll- Hypaque gradients)
8. Plastic racks to hold 50 ml conical tubes – 3
9. Aluminum foil; Red, green, yellow color coded masking tapes (1 inch wide) – 1 roll each
10. Black marker pens and self adhesive labels;

11. Parafilm
12. 50 ml conical centrifuge tubes; 16 x 100 mm round bottom disposable plastic tubes
13. Pasteur pipettes (glass or plastic); 5 and 10 ml graduated, serological pipettes
14. Automatic pipet or vacupet
15. Hydrometer (Fisher Sci.,Co.) (To determine specific gravities)

Chemicals

1. Methyl Cellulose-15 (15-Centipoises), Fisher Sci., Co.
2. Sodium Chloride (NaCl), Fisher Sci., Co.
3. Deionized distilled water (lab)
4. Hypaque (Winthrobe laboratories)
5. Ficoll-400 (Sigma ; MW 400,000)
6. EDTA-Na_2
7. Na_2HPO_4

Reagents

Name	Chemical Formula	Acceptable Grade	Health/Safety Pre-caution	Source	How to Prepare	Acceptable Performance	Storage
Methyl Cellulose (1%)	Not applicable	Not applicable	Non-toxic	In-house	See Prep. of Reagents	Should dissolve completely	Refrig. (4° C)
Physiological saline (0.9%)	Not applicable	Not applicable	Non-toxic	In-house	See Prep. of Reagents	Should dissolve completely	To be prepared fresh
Ficoll (9%)	Not applicable	Not applicable	Non-toxic	In-house	See Prep. of Reagents	Should dissolve completely	To be prepared fresh
Hypaque (50%)	Not applicable	Not applicable	Non-toxic	Commercial	Ready to use	Should give correct sp. gravity	To be used fresh
Ficoll-Hypaque sp.grav. 1.08 (lighter gradient)	Not applicable	Not applicable	Non-toxic	In-house	See Prep. of Reagents	Should give correct sp. gravity	Refrig. (4° C)
Ficoll-Hypaque sp.grav.1.12 (heavier gradient)	Not applicable	Not applicable	Non-toxic	In-house	See Prep. of Reagents	Should give correct sp. gravity	Refrig. (4° C)

Preparation of Reagents

1. Physiological Saline (0.9%); to prepare 500 ml
   a. Keep the 500 ml glass beaker ready.
   b. Using the weighing cup and analytical balance, weigh 4.5 gm of sodium chloride.
   c. Place into the beaker and using the 100 ml measuring cylinder, add 500 ml of distilled water.
   d. Using the magnetic stirrer, mix thoroughly until clear solution is obtained.
   e. Cover temporarily with aluminum foil and label on the outside of the beaker using the marker pen.
2. Methylcellulose-15 (1.0%): to prepare 500 ml
   a. Keep the 1 liter beaker ready.
   b. Using the weighing cup and analytical balance, weigh 5 gm methylcellulose-15 and place into the 1 liter beaker.
   c. Using the measuring cylinder, add 500 ml of the above prepared physiological saline slowly.
   d. Keep on the magnetic stirrer and stir until clear solution is obtained.
   e. If the solution is not clear on the same day of preparation, remove the beaker from the stirrer and keep inside the refrigerator for a day or two. *(Note: This is needed since sometimes 2 days are needed for all methylcellulose to dissolve and to get a clear solution).*
   f. When the solution is clear, distribute into the 50 ml conical centrifuge tubes (total 10 tubes) and label as:

   Reagent: Methylcellulose-15
   Conc. 1%
   Procedure: In House
   Prep. Date: ----------
   Expiration date:----------
   Made By:----------

g. Sign and enter the date of preparation and enter approximately one year for the date of expiration. In the author's lab methylcellulose had remained usable for more than one year of preparation.
h. Use color coding masking tape on the top as well as on the tube for easy identification.
i. Store all the tubes in a rack and keep at 4° C in the refrigerator.
j. Log in the appropriate log book.

3. Preparation of Ficoll-Hypaque double density gradients:
   a. Prepare 9% Ficoll-400 (total volume 500 ml) first as follows:
      1) Using top loading balance and weighing cups, weigh accurately 45 gm of Ficoll-400 and place into the 1 liter glass beaker.
      2) Using the 500 ml volumetric flask, add 500 ml of distilled water slowly and stir using the magnetic stirrer until a clear solution is obtained. Usually 2-3 hours are required for Ficoll to dissolve completely. Using the marker pen, temporarily label on the outside of the beaker as Ficoll (9%)
   b. Keep Hypaque-50 (commercial) ready.
   c. Prepare 33.9% Hypaque as follows: (to prepare 100 ml)
      1) Take a 100 ml volumetric flask.
      2) Using a 10 ml serological pipet, add 67.8 ml of Hypaque-50 in to the volumetric flask.
      3) Add distilled water carefully up to the 100 mark. Mix gently.
      4) Using the marker pen, label temporarily on the outside of the flask.
   d. Now prepare the Ficoll-Hypaque double density gradients as follows:
      1) Using appropriate volumetric flasks and serological pipettes, add 9% Ficoll, 50%Hypaque and 33.9% Hypaque in the following proportions:

	Ficoll-Hypaque (1.08 specific gravity) LG	Ficoll-Hypaque (1.12 specific gravity) HG
Beaker	500 ml	500 ml
Ficoll (9%)	240 ml	200 ml
Hypaque-50	None	100 ml
Hypaque-33.9%	100 ml	None

      2) Mix gently using a glass rod.
      3) Determine the specific gravity using the hydrometer.
      4) When the specific gravity is correct, distribute into several 50 ml conical centrifuge tubes.
      5) Label using the self adhesive label as follows:

Reagent: Ficoll-Hypaque Lighter Gradient (LG) **Conc.** Specific gravity / 1.08 **Procedure:** In House **Prep. Date:** ---------- **Expiration date:**---------- **Made by:**----------	**Reagent:** Ficoll-Hypaque Heavier Gradient (HG) **Conc.** Specific gravity / 1.12 **Procedure:** In House **Prep. Date:** ---------- **Expiration date:**---------- **Made by:**----------

      6) Use color coding masking tape on the top as well as on the tube for easy identification.
      7) Sign and date and store in a rack in the refrigerator at 4° C.
      8) Log in the appropriate log book.
      9) Do QC by taking a normal donor and ascertain the performance of the gradients in separation of granulocytes.

4. EDTA-Buffer
   a. Keep the 1 liter beaker ready.
   b. Accurately weigh the following chemicals and place into the beaker.
      Na_2HPO_4 2.6 gm
      Na_2-EDTA 3.0 gm
      NaCl 8.5 gm
   c. Add one liter of distilled water using a volumetric flask and mix using the magnetic stirrer.
   d. Warm the solution till you get a clear solution.
   e. Label the beaker, cover with aluminum foil and cool the solution by storing at room temperature overnight.
   f. Next day, measure the pH of the EDTA-buffer. The pH should be 6.8. When all the chemicals are weighed accurately and dissolved exactly in 1 liter of distilled water, the pH remains 6.8. If the pH is between 6.8-7.0, the buffer can be used. If the pH is not within this range, discard and prepare fresh.
   g. When the desired pH is obtained, distribute into two 500 ml bottles and store at room temperature.

h. Label as described above for the other reagents.
i. Enter in the reagent log book.

Instrumentation/Special Equipment

1. Analytical balance
2. Magnetic stirrer
3. Standard Table top centrifuge
4. Vortex mixer
5. Top loading balance
6. pH meter

Calibration

1. Table top centrifuge should be calibrated for its accuracy of speed once in six months. This can be done by biomedical personnel or contracted out. A signed report should be maintained in the lab.
2. The pH meter should be checked once in six months for its performance using standard solutions.

Quality Control

1. The pH of the EDTA-buffer should be checked twice a month and entered in the QC log book.
2. For methylcellulose-15 and Ficoll-Hypaque gradients, as soon as the reagents are freshly prepared, performance should be checked using 2-3 normal donors and entered in the log book. After this initial recording, a log should be maintained once a month for the performance of the reagents whenever a specimen is processed. In suspected circumstances, a special QC may be performed using normal donors. If needed fresh reagents may be prepared.
3. The isolated cells should be checked for viability and for any non-specific aggregation using a light microscope. If any cell aggregation is found, the pH of the buffer should be checked and adjusted if found incorrect.

Procedure

1. Perform all steps at room temperature.
2. Use hand gloves and water-proof lab coat before starting the procedure.
3. Use the protective shield that is available in the work area.
4. Bring one aliquot each of 1% methylcellulose, Ficoll-Hypaque-1.08 and 1.12 and EDTA-buffer to room temperature.
5. Take one 16 x 100 mm plastic disposable test tube for each 7-10 ml of blood sample and mark accordingly.
6. Transfer blood from one specimen into one tube (7-10 ml).
7. Using 5 ml serological pipet, add 2.5 ml of 1% methylcellulose to each tube, cover with parafilm and mix by gentle inversion.
8. Centrifuge at 30 x g (250 rpm) for 7-10 min.
9. Using pasteur pipet, aspirate all the supernatant leukocyte rich plasma (LRP) into a 16 x 100mm test tube and discard the red cells. This step is for convenience and to prevent leukocytes from settling down while preparing gradients.
10. Prepare double density gradients: In a 16 x 100 mm test tube, using 5 ml serological pipet, aspirate 3 ml of the HG and gently place in the tube.
11. Using another 5 ml serological pipet, aspirate 3 ml of the LG and gently overlayer on top of the HG without disturbing the lower gradient.
12. Using a 10 ml serological pipet, aspirate the LRP and overlayer gently on top of the LG.
13. Balance the tubes using the top loading balance, and centrifuge at 1650 x g (3000 rpm) for 20 min. If the separation is not satisfactory, centrifuge for a total of 30 min.
14. Following centrifugation, using a pasteur pipet, carefully aspirate the separated plasma and the first layer containing the platelets and lymphocytes. Discard the aplasma. Do not disturb the second layer. One can use the platelets and lymphoctytes for absorption purposes.
15. Now using another Pasteur pipet, aspirate the second granulocyte layer (leaving the red cell button) and transfer into another 15 ml graduated conical centrifuge tube.
16. Add buffer and make the total volume to 12-15 ml. Mix by gentle inversion, recentrifuge at 1500 rpm for 3 min and discard the supernatant. This step is needed to remove the gradient and to pellet the granulocytes.
17. Gently vortex to loosen the button, add 2 ml of buffer and centrifuge at 1500 rpm for 1 min. This step is needed to further wash the cells.
18. Discard the supernatant by decantation.
19. Suspend the cell button in appropriate buffer by gently adding up to the 1 ml mark, vortex and determine the cell concentration.
20. Adjust the cell concentration as desired for the assay (agglutination, immunofluorescence or cytotoxicity).

Calculations

Not applicable

Results and Interpretation

1. After the centrifugation, the supernatant plasma should be clear. This indicates that all the cells in the LRP have passed through the gradients and that the centrifugation was satisfactory.
2. Two distinct white cell layers should be obtained. The mixture of platelets and lymphocytes form a clear layer on top of the lighter gradient, i.e., on the interface between the plasma and the lighter gradient.
3. The granulocytes form a clear second layer on top of the heavier gradient, i.e., on the interface between the lighter and the heavier gradient. There should not be any lymphocytes trailing below the first layer. The contaminated red cells should form a small button at the bottom of the centrifuge tube. There should not be any red cells in suspension below the second layer. Red cells will contaminate the granulocytes and hypotonic lysis will be needed to eliminate these red cells. Avoid red cells! When you get a clear cell separation, this indicates that the method of layering and the centrifugation are satisfactory and the specific gravities of the gradients are accurate. Thus, one should expect granulocytes to be both viable and pure (more than 98%).

Procedure Notes

1. Methylcellulose may not form a clear solution on the same day. Overnight storage at 4° C may be necessary.
2. Sterility is not required since the solution is stored at 4° C and the assay is done on the same day.
3. When fungal or bacterial growth is observed after a prolonged period, discard and prepare fresh reagents.
4. While preparing the gradients, if accuracy is strictly maintained in weighing and measuring the water etc., the desired specific gravity is generally obtained.
5. When granulocytes are separated from large number of normal donors, particularly for screening the sera in a cell-panel assay, the author's method of layering the gradients4 is found most suitable.

Limitations of Procedure

1. In some patient specimens, all the red cells may not be contained in a clear button at the bottom of the tube. This can also be observed from specimens more than 18 hours of post-collection. You can extend the centrifugation time to resolve this.
2. In rare circumstances, lymphocyte trailing may also be observed contaminating the purity of granulocytes. You can try to shorten the centrifugation time.
3. 45-60 min are needed to isolate granulocytes from a single specimen.
4. If granulocytes are to be isolated from large numbers of donors for cell-panel assays, longer preparation time is required, particularly for layering the individual gradients and the plasma. This period can be shortened by following the author's improved method of layering the gradients.

References

1. Clay M and Kline WE, Detection of granulocyte antigens and antibodies: Current Perspectives and Approaches, In: *Current Concepts in Transfusion Therapy*, Garratty G, ed.,.American Association of Blood Banks, Arlington, pp 184-264, 1985.
2. English D and Anderson BR, Single-step separation of red blood cells, granulocytes and mononuclear leukocytes on discontinuous density gradients of Ficoll-Hypaque. J Immunol Methods 5: pp 249-252, 1974.
3. Lalezari P, Khorshidi M and Petrosova M, Autoimmune neutropenia of infancy. J Pediat 109:pp 764-769, 1986.
4. Madyastha PR, Madyastha KR, Wade T and Levine DH, An improved method for rapid layering of Ficoll-Hypaque double density gradients suitable for granulocyte separation. J Immunol Meth 48:pp 281-286, 1982.
5. Madyastha PR and Glassman AB, Characterization of neutrophil agglutinins in primary autoimmune neutropenia of early childhood. Ann Clin Lab Sci 18: pp 365- 373, 1988.
6. McCullough J, Clay M, Press C and Kline W, Granulocyte Serology: A Clinical and Laboratory Guide. Amer Soc Clin Path, pp 7-11,1988.
7. McCullough J, The clinical significance of granulocyte antibodies and in vivo studies of the fate of granulocytes. In: *Current Concepts in Transfusion Therapy*, Garratty G, ed., American Association of Blood Banks, Arlington, pp 125-182, 1985.

Assessment of Cell Preparations: A. Viability and B. Purity

Mary S. Leffell

Background and Principles

Until recently, serologic typing by the micro-lymphocytotoxicty assay and cellular testing by variations of the mixed lymphocyte culture have been the standard methods for histocompatibility laboratories and their use has been stipulated in both Federal and ASHI standards. Because these procedures require viable lymphocytes, it has been an inherent part of appropriate quality control to monitor the viability of cell preparations. Assessment of the purity of cell preparations has also been required to be able to interpret spurious reactions due to contaminating cells in lymphocyte preparations. As tissue typing moves into the next millennium, many laboratories have, or are, moving to molecular typing and are also beginning to use methods other than cytotoxicity for evaluation of HLA-specific antibodies. Notably, in August 1998, the Health Care Financing Administration ruled that molecular typing methods and enzyme-linked immunoassays were acceptable alternatives to the cell-based assays. While different technologies may totally replace the older immunologic assays and obviate the need for quality control and assurance of cell preparations in histocompatibility laboratories, serologic typing remains a cost effective method, particularly for class I typing. Moreover, as yet, there is no acceptable alternative to the lymphocyte crossmatch in histocompatibility assessment for transplantation. Perhaps one of the most important uses of the older immunologic assays will be to determine which alleles are immunologically relevant. As long as any histocompatibility assay depends upon cell preparations separated from peripheral blood or other tissues, there will continue to be a need for the procedures described in this section. Techniques for assessment of viability and purity of cell preparations must be incorporated into typing, screening or crossmatch assays as integral quality control. The purity of cell preparations should also be verified and documented as part of the fundamental quality assurance required for the development and implementation of cell isolation procedures.

The importance of verifying cell viability and purity is apparent in the ASHI standards. Eighty percent viability is the minimal acceptable level for class I and II serological typing and for cellular testing (E4.321, F4.321, and G1.100, ASHI Standards, August 1998). There is no absolute minimal standard for cell purity, because the degree required may vary with the procedure being used. For class II serological typing using enriched B lymphocyte suspensions, the proportion of B lymphocytes in each cell preparation must be confirmed and should be at least 80% (F4.420). Class I serologic typings may be performed on either a suspension of mixed mononuclear cells or T lymphocyte-enriched preparations and no minimal level for purity is given (E4.420). For antibody screening and crossmatching with separated T & B cell preparations, consideration must also be given to the degree of cell purity. While the standards do not give an exact percentage of purity for B or T enriched suspensions for crossmatches or antibody screening, the level of 80% required for other procedures seems a reasonable minimum. In fact, with commercially available reagents, levels >90% are reproducibly obtainable. If typing, antibody screening, or crossmatching is performed on cell populations other than lymphocytes, e.g., monocytes, amniotic cells, cultured cell lines, or endothelial cells, these cell preparations should also meet the minimal 80% purity level. For fluorescence techniques, which utilize mixed cell populations, no set standards of acceptable purity exist; however, prudent application of these techniques should incorporate assessment and documentation of the percentage of cell types present.

A. Assessment of Viability

Purpose

The purpose of monitoring cell viability is patently obvious, since it would be illogical to try to perform cytotoxicity testing or a MLC with non-viable cells. Routine documentation of the viability of cell preparations, however, serves two other important functions. If the viability of cell suspensions are marginally acceptable, knowledge of this fact may aid in the interpretation of spurious typing reactions or questionable crossmatch results. Secondly, monitoring the viability of cell preparations over time can indicate if problems develop with the isolation techniques.

Vital dye exclusion has become the generally accepted procedure for determining cell viability in histocompatibility laboratories because it is quick and cost effective. The assay depends on the ability of viable cells to exclude dyes such as trypan blue, eosin, nigrosin or ethidium bromide. In contrast, dead or dying cells, whose cells membranes become permeable, take up these dyes into the cytoplasm and nucleus. Trypan blue is most commonly used and is applicable to most any cellular preparation, including cultured cell lines and amniotic cells.

Reagent

Trypan Blue

1. 1% Stock Solution
   a. Dissolve 1 g trypan blue in 100 ml distilled H_2O.
   b. Filter (5-10 µ or Whatman #1) or centrifuge and store at 2-8° C
2. Daily Working Solution – 0.3%
   a. 3 ml stock solution
   b. 7 ml balanced salt solution/medium (PBS, HBSS, EDTA-barbital buffer, McCoy's RPMI)
   This solution should be prepared fresh daily.

Instrumentation/Special Equipment

1. Hemacytometer appropriate to the microscope style (inverted or regular)
2. Microscope

Calibration

Not Applicable

Quality Control

Standard reagent and equipment quality controls should be followed and must be documented. See chapter on Quality Control in this manual.

Procedure

1. Mix equal volumes of cell suspension and 0.3% trypan blue.
2. Load cell/dye suspension onto a hemacytometer. Use of the hemacytometer allows simultaneous determination of viability and adjustment of cell concentration.
3. Allow lymphocytes to settle for 1-2 min. This time period for cells to settle on the hemacytometer is critical for accurate assessment of both viability and lymphocyte yield. If examined immediately, small lymphocytes in suspension will be missed when the microscope is focused on the hemacytometer surface.
4. Count a minimum of 100 cells and determine the percentage of viable cells. Viable cells excluding the dye will be well rounded and refractile. Conversely, dead cells will be stained and flattened on the hemacytometer surface.

Calculations

Not Applicable

Results and Interpretation

Pretest viability should be determined and recorded for serological and cellular typing procedures. Additionally, for class I and II serologic typing it is advisable to record post-test viability. Checking viability pre- and post-testing can control for cell preparations that may be compromised by age or adverse storage or handling. As stated above, ASHI standards recommend 80% viability as the minimal acceptable level. For all histocompatibility procedures, but especially crossmatches and enriched-B lymphocyte DR typings, viability greater than 90% is preferable. Cell preparations with less than acceptable viability should not be used. A fresh cell suspension should be prepared (from another, fresh specimen if necessary) or alternatively, various "clean-up" techniques may be used to remove dead cells.

Procedure Notes

Variations

1. Alternate vital dyes.
   As mentioned above, other vital dyes such as eosin or ethidium bromide may be used, but whatever dye is used, the procedure should be standardized and in routine use.
2. Viability of monocytes and granulocytes.
   The above trypan blue procedure may be used to assess the viability of monocyte or granulocyte preparations with the following modifications
3. Working trypan blue suspension – 0.2%.
   Prepare as above in any osmotic suspension medium and reduce the trypan blue concentration to 0.2%. Additionally, because monocytes and granulocytes are phagocytic cells, it is critical to be certain that the solution is free of dye particles by filtration or centrifugation. Excess particles will be ingested by phagocytes and give spuriously high levels of stained cells.

4. Setting Time – After loading the hemacytometer, allow only 2 min for the cells to settle. With longer setting times both monocytes and granulocytes will begin to adhere to the hemacytometer. During this process they will "flatten" out on the hemacytometer surface and will result in a falsely elevated estimate of dead cells.

Limitation of Procedure

Vital dye exclusion is not a sensitive measure of cell viability and cells may be in an advanced state of degeneration and still be able to exclude dye. Checking viability pre- and post-testing can control for cell preparations that may be compromised by age or adverse storage or handling.

B. Assessment of Purity

Purpose

There are numerous reasons why it is so critical to ascertain the purity of cell preparations utilized in histocompatibility testing. It has long been recognized for class I serologic typing that the mononuclear preparations should be free of other hematopoietic cells, i.e., platelets, granulocytes, and erythrocytes. Because granulocytes and platelets express class I antigens, they can compete with lymphocytes for reactivity with reagent antibody, leading to false negative typing reactions. Erythrocyte contamination may cause another kind of problem. Red cells under low-power microscopy resemble small lymphocytes and may lead to false negative results if the contamination level is high. For class II serologic typing, excess contamination of T lymphocytes and monocytes may cause erroneous results regardless of technique, i.e., enriched B lymphocyte or 1-2 color fluorescence. A preponderance of T cells in a cell preparation for class II typing often results in false negative reactions, whereas excess monocytes may cause extra reactions or high background readings. Conversely, excess B lymphocytes or monocytes can cause equivocal reactions in T enriched crossmatches if the serum being tested contains any class II antibodies. Finally, it should be remembered that cell purity is not just a critical factor for serologic typing procedures but is just as important in cellular procedures. Granulocyte contamination in mononuclear cell preparations for MLC's is a prime example. Because granulocytes are end cells, they will die during the first 1-2 days of culture, therefore, cultures adjusted to concentrations based on total cells will give erroneously low results if there were contaminating granulocytes in the cell suspension.

1) Purity Assessment By Phase Contrast Microscopy

Morphological assessment of cell preparations by phase contrast microscopy is a routinely used method for checking cellular purity for serologic typing and often for cellular typing. Under phase contrast, lymphocytes will appear as well-rounded cells, larger than erythrocytes, and smaller than the more irregularly shaped, "grey" granulocytes and monocytes. Platelets are the smallest of all cells and often are found in aggregates.

Reagents and Supplies

Glass slides and coverslips

Instrumentation/Special Equipment

Phase Contrast Microscope

Calibration

No special calibration is needed.

Quality Control

Phase rings must be aligned prior to use.
Standard reagent and equipment quality controls should be followed and must be documented.
See chapter on Quality Control in this manual.

Procedure

A drop of cell suspension is mounted under a coverslip and examined under phase contrast.

Calculations

Not Applicable

Results and Interpretation

Generally, a subjective evaluation is used for judging cell preparations. Contamination with platelets or granulocytes is usually obvious and there is no need to perform actual cell counts. If the cell preparation is to be used for serologic typing, it must be further purified before use.

Procedure Notes

Problems can arise when contamination is marginal and an inexperienced person is evaluating the cell suspension. Learning to evaluate the literal "shades" of difference in cell morphology requires experience; therefore, it is recommended that senior lab personnel be the ones responsible for assessing purity by phase microscopy.

2) Purity Assessment Utilizing Control Sera on Typing Trays

While phase microscopy is a good method to screen for gross contamination of cell preparations, the best approach for routine evaluation in serologic testing is the utilization of control sera specific for different cell populations. While positive and negative control sera are sufficient for most class I typings, proper evaluation of class II typings or specific cell crossmatches requires control sera specific for cellular lineage, including T and B lymphocytes, monocytes and granulocytes. Most commercial suppliers now include cell type controls on class II trays. Bulk sera controls are also available for use on locally prepared class II typing assays or for inclusion on crossmatch trays.

Reagents and Supplies

1. Glass slides and coverslips.
2. **SUGGESTED SERA CONTROLS FOR SEROLOGIC TYPING**
   Currently there are several excellent commercial sources for different control sera. A list of some suppliers is included at the end of this section. One cautionary reminder: as many of the available reagents are monoclonal antibodies (MoAbs), it should be remembered that not all MoAbs fix complement. Therefore, the ability of a MoAb to mediate cytotoxicity should be considered in selecting controls.
   a. Anti-T cell: There are both polyclonal and monoclonal antisera available for use as positive controls for T lymphocytes. Antisera generated against T lymphocyte differentiation antigens by immunization in other species, such as anti-thymocyte globulin, can be used. Among the MoAbs, a complement fixing antibody with specificity for CD3 is a good alternative.
   b. Anti-B cell: Many B cell control sera currently in use for serologic typing are directed against framework or monomorphic DR determinants. Such reagents are excellent indicators of B cell numbers but may also react with monocytes and activated T cells in the preparation. Use of a MoAb with another anti-B lymphocyte specificity, e.g., CD19 or CD20, may allow more accurate assessment of B lymphocytes, particularly if DR expression is reduced.
   c. Monocyte/Granulocyte Controls: A good panel of controls will also include sera with specificity for monocytes or granulocytes. Because monocytes and granulocytes are both members of the myeloid cell lineage, many of the commercially available reagents react to some extent with both cell types. Therefore, the specificity(ies) of the control serum needs to be remembered not only when selecting controls, but also in interpreting results. Differentiation markers for myeloid cells are discussed further below.

Instrumentation/Special Equipment

Inverted Microscope (with mercury or xenon lamp and appropriate exciter/barrier filters if using fluorescent dyes).

Calibration

No special calibration is needed.

Quality Control

Standard reagent and equipment quality controls should be followed and must be documented. When using any MoAb, it is also wise to titrate the reagent against known cells to determine the optimal working dilution. See section on Quality Control in this manual.

Procedure

Appropriate serological testing using standard procedures (see Serology section in this manual).

Calculations

Not Applicable

Results and Interpretation

Table 1 gives examples of reaction scores and the expected percent of given cell populations based on the total number of cells plated. The examples given are for lymphocyte preparations from peripheral blood. Remember that these sera controls are assessing the cellular composition of the prepared specimen, and it is therefore important to evaluate the whole population. This becomes critical in methods such as 1 or 2 color fluorescence class II typings, where the final reaction scores are based on just the B lymphocytes in the preparation.

Procedure Notes

It should be remembered, despite the value of controls such as those suggested above, that serological typings or crossmatches have extra, inherent controls in the reactions of cells with typing sera or patient sera. It is always a good idea to evaluate the overall patterns of reactivity of a given tray. For example, if you are performing a T cell crossmatch against sera with IgM anti-B cell reactivity and your prep has contaminating B lymphocytes and/or monocytes, extra 2-4 reactions might alert you to this possibility, regardless of how your control sera reacted. Another example might be cases of class II typing where your cell preparation includes B lymphocytes with reduced DR expression. In such cases, you may see weak reactions with DR sera, but strong reactions with DQ reagents. The importance of controls, both intentional and inherent, should not be underestimated. Their proper use and interpretation not only validate the results, but also provide direction as to possible solutions for the problem.

Limitation of Procedure

Whenever antibodies are used in a procedure, it is wise to determine the source of the antibody (what species produced the serum) as well as the target of the antibody (what the antibody will bind to). It is critical to match the target of the antiserum to the desired cell population (i.e., *anti-human*-globulin to a *human* target cell, or *anti-human IgM* to a *human* cell producing *IgM*). Otherwise antigen recognition may not occur, sufficient binding may not take palce, and the desired end effect (fluorescence or complement-mediated lysis) may not be accomplished.

Table 1: Examples of Control Sera Reactions and Their Interpretation

Cell Preparation	B Cell (Non-DR)	B Cell (Anti-DR)	T Cell	Monocyte	Granulocyte	Interpretation
1. T Lymphocyte	1[a] ≤ 10%[b]	1 ≤ 10%	8 80-100%	1 ≤ 5%	1 ≤ 5%	Ideal enriched T lymphocyte preparation
2. B Lymphocyte	8 80-100%	8 80-100%	1 ≤ 10%	1 ≤ 10%	1 ≤ 5%	Ideal enriched B lymphocyte preparation
3. B Lymphocyte	8 80-100%	2-4 20-50%	1 ≤ 10%	1 ≤ 10%	1 ≤ 5%	Adequate numbers of B cells, but reduced DR expression
4. B Lymphocyte	4-6 40-70%	4-6 40-70%	2-4 20-50%	1 ≤ 5%	1 ≤ 5%	Significant T cell contamination of B lymphocyte preparation
5. T Lymphocyte	2 10-20%	2 10-20%	6 50-70%	2 10-20%	1 ≤ 5%	Contamination of T cell preparation with B cells and monocytes
6. T and B Lymphocyte Preparation	2 10-20%	2 10-20%	6-8 70-80%	1 ≤ 5%	1 ≤ 5%	Ideal proportions for a mixed preparation from peripheral blood
7. T and B Lymphocyte Preparation	1 ≤ 5%	1 ≤ 5%	8 85-95%	1 ≤ 5%	1 ≤ 5%	Low % of B lymphocytes. Such preparations should not be used for 1 or 2 color fluorescence DR typing.

[a] Cytotoxicity Score Using ASHI Recommended Standards

[b] Proportion of Cell Type

3) Assessment of Cellular Preparations During Development of New Procedures

Immunomagnetic beads have widespread use in the preparation of purified or enriched cell preparations for typing and/or crossmatching. During the development and validation of this or any other purification method, the purity of the cell suspensions should be determined, as well as monitoring the efficacy of the method, once instituted on a daily basis. While controls, such as discussed in the previous section, are adequate for daily use, more quantitative analysis is preferable during development of a new procedure. Staining of different cell populations with monoclonal antibodies (MoAbs) followed by flow cytometric analysis is an ideal and commonly used method for analyzing cell purity. By using two or more markers for different populations in multicolor cytometric analysis, the distribution of leukocyte subpopulations can be determined. As there is a thorough discussion of the principles of flow cytometry in another section of this manual, the procedures will not be discussed here. For laboratories that do not have access to a flow cytometer, direct visualization of appropriately stained cells by fluorescence microscopy can be used. The MoAbs suggested below can be applied in either approach; however, if fluorescence microscopy is used, MoAb:fluorochrome conjugates with higher specific activity than those used in flow cytometry are recommended.

Reagents

1. **MoAbs** to leukocyte differentiation antigens or other surface markers. These reagents may be obtained from several commercial surfaces (see appended list) and most are available as direct conjugates with fluorescein isothiocyanate (FITC) and/or phycoerythrin (PE).
   a. **B lymphocytes:** MoAb against CD19, CD20 and/or CD21. All are relatively specific for B lymphocytes. CD19 is a transmembrane glycoprotein that is part of the B cell antigen receptor complex. CD20 is thought to be a phosphoprotein that functions as a calcium-channel subunit and CD21 is a receptor (CR2) for the complement component C3d.

      Polyvalent anti-Human Ig $F(ab')_2$ fragment. Surface Ig, the B lymphocyte antigen receptor can also be used as a B cell marker. However, because other leukocytes, such as monocytes, can bind serum immunoglobulins through their Fc receptors, the use of an $F(ab')_2$ reagent is recommended to ensure specificity for B lymphocytes.
   b. **T lymphocytes:** CD3, a complex of glycoproteins associated with the T cell receptor, is the most specific T lymphocyte differentiation marker. CD4 and CD8 can be used to define the two major T lymphocyte populations.
   c. **Monocytes:** As discussed above, there is currently no MoAb with absolute specificity for monocytes. Most available reagents react to some extent with other myeloid cells such as granulocytes and NK cells. If this is kept in mind, these MoAbs can be used to estimate the degree of monocyte contamination or enrichment. CD14, a lipopolysaccharide receptor, or CD91, a receptor for α_2-macroglobulin, are two suggested monocyte markers.
   d. **NK cells:** CD16 is a Fc receptor which is often used for defining NK cells. It is, however, also expressed on activated monocytes and macrophages. CD56, also known as NKH1, is a cell adhesion molecule and may be expressed on some lymphocytes as well as NK cells.
2. **Wash media** – Hank's balanced salt solution (HBSS), RPMI, or phosphate buffered saline (PBS) with 0.5% bovine serum albumin (BSA) are all suitable.

Procedure for Fluorescence Microscopy

1. Place 1-3 x 10^6 prepared cells into a 10 x 75 mm polystyrene tube and wash one time with 0.5% BSA-medium.
2. After centrifugation (300 x g), discard wash supernatant, and add 5-40 µl of MoAb- FITC. Check manufacturer's recommendation for a starting point to determine the optimal amount of conjugated MoAb to use.
3. Incubate cell-MoAb mixture for 30 min at 2-8° C.
4. Following incubation, wash cells 3 times with 0.5% BSA-medium. Resuspend final cell pellet in 5-100 µl of medium and mount on a slide with coverslip.

   Note: Increased sensitivity can be achieved by indirect or double antibody staining. For the first incubation, use an unconjugated MoAb. Wash as above and then perform the second incubation with a FITC-conjugated antiglobulin with specificity for the species of the MoAb source (usually mouse). Again, wash the stained cells and proceed to step 5.
5. Count at least 200 cells and determine the percentage of stained cells.

Calculations

Simple standard percentage calculations are all that are required.

Results and Interpretation

Fluorescence Microscopy

It is best to examine stained cells as soon as possible since fluorescence intensity is lost with time. A high resolution oil immersion objective is recommended. Appropriately stained cells will exhibit a ring or "cap" of membrane fluorescence. Dead cells in the preparation will exhibit diffuse, cytoplasmic staining. With some cell preparations, notably B lymphocytes stained for surface Ig, capping and subsequent endocytosis of surface markers may cause false negative results if the cells are not examined immediately. To prevent capping, 0.01% sodium azide may be added to the wash media used after antibody incubation.

Procedure Notes

Due to the fact that incident light may quench fluorochromes, it is best to perform the cell-MoAb incubation in the dark.

Limitation of Procedure

Fluorescence microscopy is limited by the same factors that affect any antibody mediated assay, e.g., the specificity and affinity of the antibody, as well as the specific activity of the reporter molecule. In practice, fluorescence microscopy is often further hindered by higher degrees of background staining resulting from cell preparations with compromised viability and/or antibody preparations with immunoglobulin aggregates.

4) Assessment of Monocytes

Because, as mentioned above, there is no truly specific cell surface marker suitable for monocytes, two additional methods for assessing monocytes are discussed briefly.

LATEX INGESTION

Principle

This procedure relies on the phagocytic ability of monocytes and is relatively simple to perform.

Reagents

1. 5% suspension of 1.1µm latex particles in HBSS with 1% BSA.
2. Latex particles should be pre-washed by centrifugation several times in a medium before use. Latex particles may be obtained from: Dow Chemical, Diagnostics Division, Indianapolis, IN 46206.
3. Prepared cell suspension adjusted to 1 x 10^6 cells/ml 1% BSA-HBSS.

Procedure

1. Add 10 µl of a latex suspension to 1 ml of cell suspension.
2. Mix and incubate for 30 min at 35-39° C.
3. Following incubation, wash 3-4 times with 1% BSA-HBSS using low-speed centrifugation (200 x g) for 10 min so that free, uningested latex will not pellet and will be discarded in wash supernatant. Check supernatant for free latex to determine when washing is complete.
4. Resuspended final cell pellet in 50-100 µl of a 1% BSA-HBSS or suitable medium and mount under a coverslip.
5. Examine by phase microscopy with an oil immersion objective. Phagocytic cells will have 3 or more ingested latex particles. Focus up and down on each cell to determine whether or not the particles are actually ingested or are free-floating.

Results and Interpretation

The percent of the total cells examined will be an estimate of monocyte purity or contamination. Remember that any granulocytes in the preparation will also ingest latex particles.

α-NAPHTHYL ACETATE ESTERASE

Principle

This enzyme is considered to be the most specific histochemical marker for monocytes because the enzyme is virtually undetectable in granulocytes. Lymphocytes may exhibit some activity, but the staining pattern is easily distinguishable from that of monocytes. In performing the test, fixed cellular smears are incubated with substrate, a-naphthyl acetate, in the presence of a diazonium salt. The enzyme activity hydrolyzes ester bonds yielding free naphthol compounds which couple with the diazonium salt, leaving dark brownish-black deposits at the site of enzyme activity.

Reagents

Reagents may be purchased in kit form from Sigma Diagnostics, St. Louis, MO 63178. Alternatively, a detailed procedure including reagent preparation has been outlined by Sun et al.[5] Hematology services also generally offer this and other esterase procedures. Because reagent preparation is involved, unless this assay is to be used extensively, it is recommended to utilize the services of a hematology laboratory. Only the basic steps and principles of this assay will be outlined here.

Procedure

1. Smears, or preferably cytocentrifuge preparations of cells to be tested, are fixed for 30 seconds in a fixative such as cold buffered-formol acetone.

2. After fixation, the smears are washed with distilled water and allowed to air dry. Fixed specimens may be stored at room temperature for several weeks without loss of activity. Unfixed smears should only be stored for a few days prior to staining.
3. Fixed smears are then incubated with the substrate solution at room temperature for 45 min. Following incubation, slides are rinsed thoroughly with water.
4. Generally, the smears are then counter-stained with methyl green or Mayer's hematoxylin for 1-5 min.
5. After rinsing and drying, smears are then mounted with a suitable mounting medium.

Results and Interpretation

Enzyme activity will appear as dark red to brownish-black deposits in the cytoplasm of monocytes. The deposition pattern will be intense and diffuse in monocytes, whereas only small deposits are occasionally seen in lymphocytes.

Suggested Commercial Sources of Cell Control Sera

There are many suppliers of control sera and this list is not intended to be comprehensive. The suppliers listed below have proven reagents currently in use in several laboratories familiar to the author.

1. Accurate Chemical and Scientific, Westbury, NY 11590, (800) 645-6264.
2. Becton-Dickinson Immunocytometry and Cellular Imaging, San Jose, CA, 95131, (800) 223-8226.
3. Biotest Diagnostic's Corporation, Fairfield, NJ 07006, (800) 522-0090.
4. Boehringer Mannheim, Indianapolis, 46250, (800) 262-1640.
5. Coulter Immunology Division, Hialeah, FL 33010. (800) 526-7694.
6. Dako, Carpinteria, CA 93013, (800) 235-5743.
7. One Lambda, Los Angeles, CA 90064, (800) 822-8824.
8. Ortho Biotech Division, Raritan, NJ 08869, (800) 322-6374.
9. PharMingen, San Diego, CA, 92121, (800) 848-6227.
10. Pel-Freez Biologicals, Rogers, AK 72757, (800) 636-4361.
11. Serotech, Bicester Oxford, OX6 OTP, England, (44) 1865-379941; Fax: (44) 1865- 373899.

References

1. Bray RA and Stempora L, Phenotyping by Immunofluorescence. In: The ASHI Laboratory Manual, 3rd ed., A Nikaein, DL Phelan, EM Mickelson, HS Noreen, TW Shroyer, eds.; American Society for Histocompatibility and Immunogenetics, Lenexa, p V.2.1, 1994.
2. Fleischer TA and Marti GE, Detection of Unseparated Human Lymphocytes by Flow Cytometry. In: *Current Protocols in Immunology*; JE Coligan, AM Kruisbeek, DH Margulies, EM Shevach, and W Strober, eds; John Wiley & Sons, Inc., New York, p 7.9.1, 1991.
3. Kadushin J, Resolution of Purity Problems. In: *The ASHI Laboratory Manual*, 2nd ed., AA Zachary and GT Teresi, eds.; American Society for Histocompatibility and Immunogenetics, New York, p 81, 1990.
4. Kidd PG and Nicholson JK. Immunophenotyping by Flow Cytometry. In: *Manual of Clinical Laboratory Immunology*, 5th ed.; NR Rose, EC de Macario, JD Folds, HC Lane and RM Nakamura, eds.; American Society of Microbiology, Washington, DC; p 212, 1997.
5. Lou CD, Cunniffe KJ and Garovoy MR, Histocompatibility Testing by Immunologic Methods: Humoral Assays. In: *Manual of Clinical Laboratory Immunology*, 5th ed.; NR Rose, EC de Macario, JD Folds, HC Lane and RM Nakamura, eds.; American Society of Microbiology, Washington, DC; p 1087, 1997.
6. Reinsmoen NL. Histocompatibility Testing by Immunologic Methods: Cellular Assays. In: *Manual of Clinical Laboratory Immunology*, 5th ed.; NR Rose, EC de Macario, JD Folds, HC Lane, RM Nakamura, eds.; American Society of Microbiology, Washington, DC; p 1080, 1997.
7. Strober W. Trypan Blue Exclusion Test of Cell Viability. In: *Current Protocols in Immunology*; JE Coligan, AM Kruisbeek, DH Margulies, EM Shevach, W Strober, eds; John Wiley & Sons, Inc., New York, p A.3B.1, 1997.
8. Stux S and Fotino M, Assessment of B cell purity. In: *AACHT Laboratory Manual*; AA Zachary and WE Braun, eds., American Association for Clinical Histocompatibility Testing, New York; p I-12-1, 1981.
9. Sun T, Li C and Yam LT: *Atlas of Cytochemistry and Immunochemistry of Hematologic Neoplasms*. American Society of Clinical Pathologists Press, Chicago; Nonspecific Esterase Reactions; pp 24 & 97, 1985.
10. van Furth R and van Zwet TL: In vitro determination of phagocytosis and intracellular killing by polymorphonuclear and mononuclear phagocytes. In: *Handbook of Experimental Immunology*; DM Weir, ed.; Blackwell Scientific Publications, London; p 36.1, 1973.
11. Winchester RJ and Ross GD, Methods for Enumerating Cell Populations by Surface Markers with Conventional Microscopy. In: *Manual of Clinical Laboratory Immunology*, 3rd ed.; NR Rose, H Friedman, JL Fahey, eds.; American Society of Microbiology, Washington, DC; p 212, 1986.

Recalcification of Plasma

Herbert A. Perkins, Nancy Sakahara and Zenaida P. Gantan

Purpose

To convert plasma into serum before use as typing reagent. Large volumes of useful typing reagents are usually obtained from donors in the form of citrated plasma, either by recovering the plasma from a standard blood bank donation of whole blood or by plasmapheresis. This plasma should be converted to serum before use as a typing reagent, since the fibrinogen of plasma will precipitate in the cold and may clot in the presence of thrombin.

Clotting of the plasma is prevented by binding calcium ions with citrate in the form of sodium citrate, ACD solution, CPD solution or CPD-Adenine solution. Recalcification of plasma to cause coagulation is accomplished by adding calcium to complex with the citrate and leave sufficient excess calcium to restore a normal ionized calcium level. Insufficient or excess calcium will delay or prevent coagulation. The optimal amount of calcium to be added will vary with the amount of citrate in relation to the volume of blood collected, the calcium binding capacity of plasma proteins and other anions and the donor's hematocrit. However, the required amount of calcium to ensure complete clotting, given sufficient time, does not have to be exactly optimal; arbitrary addition of the average amount of calcium required to restore a normal ionized calcium level almost always has the desired results.

Also, cryoprecipitable proteins are removed with the clot by storing the clotted plasma overnight in a refrigerator and then centrifuging it at refrigerator temperature.

Specimen

Citrated plasma from whole blood or plasmapheresis product.

Unacceptable Specimen

Hemolyzed or chylous plasma

Instrumentation

1. Sorvall (RC3 cold centrifuge)
2. Mettler scale
3. Refrigerator (0-5° C)
4. Ultra-low freezer (-70° C)
5. Storage vials (2 ml and 50 ml size)
6. Fenwal clips
7. Hemostat
8. 5 cc syringe with 21 gauge needle

Reagents

1. 2M Calcium chloride ($CaCl_2$) solution
2. Topical thrombin (1000 units per ml Parke Davis, Detroit, MI)

Procedure

Carry out the procedure in the plastic bag into which the plasma was originally collected to minimize the likelihood of bacterial contamination.

1. Process the plasma within six hrs of collection for best results. Alternatively, may freeze plasma within six hrs of collection and store at -20° C or below. Plasma may be stored for years prior to recalcification if maintained at -70° C. Warm to room temperature before recalcification.
2. Weigh the bag and subtract the weight of an empty bag. Assume that 1 gram = 1 ml to determine plasma volume.
3. Fill a sterile plastic disposable syringe with 1 ml 2M Calcium chloride for each 100 ml of plasma. Clamp the collection tubing of the bag immediately proximal to the seal, cut off the seal, insert the tip of the syringe into the tubing, and empty the syringe into the bag.
4. Seal the tubing electronically or with a clamp and mix the $CaCl_2$ solution into the bag by compressing the tubing repeatedly, allowing plasma to reflux into the tubing each time. Invert the unit each time to ensure complete mixing of the calcium chloride solution with the plasma.
5. Incubate the plasma at room temperature overnight.
6. Detach the clot from the walls of the bag by external manual compression. Incubate overnight at 1-6° C.

7. Centrifuge 10 min at 4000 x g at 1-6° C.
8. Express the serum into aliquots of 2 ml for retesting and 50 ml vials for storage.
9. Store sera in -70° C freezer.

Troubleshooting

If no clot has formed after step 5, add 0.1 ml topical thrombin to the bag and mix well. Incubate overnight.

If a precipitate remains after step 7, or forms after further storage in the cold, recentrifuge at 10,000 x g or higher in the cold.

Reference

Unpublished data, Irwin Memorial Blood Centers, San Francisco.

Absorption with Lymphocytes

Gary A. Teresi and Anne Fuller

Principle/Purpose

Antibodies in serum can be tested and characterized to gain insight as to their affinity, avidity and specificity. Complete characterization of the antibodies can be useful to the clinician evaluating a patient for transplantation, to the researcher investigating an immunologic process, and to the technologist investigating the activity of a patient's serum or a potential HLA typing antiserum. One simple technique to dissect antibody specificity is the absorption of serum with lymphocytes and/or B cell lymphoblastoid cell lines and retesting by lymphocytotoxicity.

An apparently polyspecific antiserum can be absorbed with lymphocytes to determine if it is a mixture of antibodies or a single antibody. As an example, a serum that reacts with HLA-A1 and -B41 by direct cytotoxicity can be absorbed with lymphocytes expressing A1 but not B41. If the serum possesses two separate antibodies, the absorbed serum may react as a "monospecific" B41 antiserum. However, if the antibody present in the serum is reacting with a determinant common to both A1 and B41 (a public determinant), than the absorbed serum will be non-reactive. One should be cautious in interpreting the latter results because direct cytotoxicity requires more than one antibody for complement activation and the serum may contain both public and private antibodies. A more sensitive assay, specifically either antiglobulin augmented cytotoxicity or flow cytometry, will detect both private and public (many which CYNAP) antibodies present in the serum. After the absorption and retesting by the sensitive technique, the results may become more interpretable.

Another application of serum absorption with lymphocytes is to remove autoantibodies, particularly where they complicate the interpretation of crossmatch tests, e.g., the so called "false positive" crossmatch.[1-8] Alloantibodies cannot be characterized readily in the presence of an autoantibody. Autolymphocyte absorption can remove autoantibody which, in turn, prevents a false positive crossmatch result and permits testing for alloantibody.

A third application has been the differentiation of antibody to T versus B lymphocytes. This has been used in resolving difficult crossmatch problems.[9]

The procedure for absorption involves incubating the serum with lymphocytes, recovering the absorbed serum and testing the serum for completion of absorption.

Incubation of the serum with lymphocytes should be done at the optimal temperature for antibody reactivity. Autoantibodies are most commonly cold reacting. Therefore, incubation of cells and serum in the absorption procedure should be performed in the cold (4° C). For investigating warm reacting B cell antibodies, the B lymphocytes and serum should be incubated at warm temperatures (22-37° C). The most important aspect of the procedure is testing to determine the efficacy of the absorption. The same initial technique that was used to identify the antibody to be absorbed must be repeated with the absorbed serum. The result will indicate if the absorption is complete or if further absorption is needed. The procedure that follows includes testing with untreated serum to allow for a parallel comparison of reactivity to determine the extent, if any, of further absorptions necessary to completely abrogate serum reactivity.

Specimen

1. Serum or recalcified plasma
2. Autologous cells or reference cells

Specimens used in this assay are unacceptable if they are handled in such a way that the identity of the specimen is in question and/or the antibody is damaged. Antibody damage, especially damage to auto- or allo-IgM, can occur if blood specimens are exposed to excessive temperature extremes.

Reagents

1. Crossmatch controls
2. Patient's serum
3. Patient's autologous lymphocytes

Instrumentation/Special Equipment

1. Centrifuge
2. Refrigerator and/or waterbath, 37° C

Procedure

Absorption of Cold Reacting Auto-T Lymphocytes

1. Isolate T lymphocytes by appropriate method (see Lymphocyte Isolation chapter).
2. Distribute 5 x 10^6 lymphocytes into each of three tubes labeled #1, #2 and #3 and 0.5 x 10^6 into tube #4. Refrigerate #2, #3 and #4 for later use. The fourth suspension of lymphocytes is for testing the completeness of absorption as described in step #17.

3. Centrifuge tube #1 at 700-1000 x g for 1 min to pellet the lymphocytes.
4. Completely discard all supernatant to minimize diluting the serum.
5. Thoroughly resuspend the lymphocytes in 200 µl of the serum to be autoabsorbed.
6. Incubate 1 hr at 4° C.
7. Centrifuge at 700-1000 x g for 1 min.
8. Centrifuge tube #2 at 4000 x g for 1 min. For efficiency combine steps 7 and 8 in a single centrifugation.
9. Discard all supernatant of #2 (as done in step #4).
10. Transfer the 1X autoabsorbed serum from the #1 tube to tube #2 and thoroughly resuspend the lymphocytes in the serum.
11. Incubate 1 hr at 4° C.
12. Centrifuge at 700-1000 x g for 1 min.
13. Transfer the 2X autoabsorbed serum to a microtube, being careful to avoid pelleted cells.
14. Prepare serial dilutions of the 2X autoabsorbed serum. (We routinely prepare dilutions to 1:4, at a minimum, regardless of the original titer.)
15. Dispense 3-5 µl mineral oil into each well of a microtest tray.
16. Place 1 µl of each of the following under the oil:
    a. Negative control
    b. Positive control
    c. Untreated serum: 1:1, 1:2, 1:4
    d. 2X autoabsorbed serum: 1:1, 1:2, 1:4
    e. Cell purity positive control (see control table)
17. Perform testing with lymphocytes saved in tube #4 using the technique used to identify the antibody, originally.

Controls

Control	Components	Expected Results	Purpose
Negative	Non-cytotoxic reagent	-	Reveals extent of nonspecific cell death
Positive	Complement-dependent anti-lymphocyte reagent	+	Demonstrates that a positive reaction will occur in the presence of lymphocytotoxic Ab
B Cell	Complement-dependent anti-B cell reagent	- (with T cells) + (with B cells)	Demonstrates proportion of B cells in cell suspension and serves as positive control when B cells are tested*
Monocyte	Complement-dependent anti-monocyte reagent	- (with T cells) - (with B cells) + (with monocytes)	Demonstrates proportion of monocytes in the cell suspension and serves as positive control when monocytes are tested.

* Use of cell specific (purity) controls will depend on the cells used in testing.

Results

Autoantibody investigations should result in positive reactivity with the untreated serum and negative reactivity with the absorbed serum indicating complete autoabsorption. Further absorption using the cells in tube #3 should be performed if the 2X autoabsorbed serum is reactive. When all autoantibody is removed, the absorbed serum can then be tested for other antibodies and/or used in crossmatching.

Procedure Notes

Troubleshooting

If the 2X autoabsorbed serum remains autoreactive, additional absorptions should be performed. If reactivity remains, the autoantibody titer may be extremely high. The clinician should be notified so that the possibility of an undetected autoimmune disease (e.g., systemic lupus erythematosus), drug reaction (e.g., procainamide), or other cause can be identified.

Common Variations

A simple variation is to change the incubation temperature. Antibodies that react optimally at 37° C should be absorbed at 37° C. The procedure, including cell isolation, should be performed aseptically to avoid microbial contamination.

The number of lymphocytes used in the absorption can be varied. The cell concentration used in the above procedure has been sufficient for removing autoantibodies from almost all patients demonstrating autoantibodies in my lab. Only a few patients with presumably an extremely high titer of autoantibodies required three absorptions for complete removal of the autoantibodies. Remember that each additional absorption step increasingly dilutes the serum since it is impossible to completely remove the medium of the cell pellet.

The above procedure is most easily applied to removal of autoantibodies. A similar procedure can be used to characterize the number and specificity of antibodies contained in a transplant patient's serum and/or an HLA typing reagent. The absorbing cells can be either lymphocytes isolated from blood, lymph node or spleen or alternatively, B lymphoblastoid cell lines. The advantages of using EBV cell lines are that many of them are class I homozygous and well characterized, they express more class I antigen than lymphocytes and, in theory, the supply is endless. In a study we did many years ago, the number of EBV cells needed to absorb one ml of a serum that had a titer of 1:4 by antiglobulin augmented cytotoxicity (AHG-CDC) was 1.0×10^6. The phenotype of the cells that are used for the absorption is critical to fully define the specificities in the serum. For example, an HLA typing reagent that reacts with B7 by direct cytotoxicity (NIH-CDC) may have panel reactivity (PRA) of >80% by antiglobulin augmented cytotoxicity and/or flow indirect binding assays. Panel reactivity suggests that the antibody is reactive with all cells that express Bw6 and a number of extra reactions. To clarify what, if any, additional antibody is present, the phenotype of the absorbing cell to try should be Bw6 positive; B7 negative. Retesting by the sensitive AHG-CDC assay after the absorption (in parallel with the unabsorbed sera) would prove that there are at least two, if not three antibodies in this serum. The public Bw6 antibody would be absorbed; the remaining reactivity would react with the cells of the 7CREG (B7,22,42,27). Potentially, the serum also contains a private B7 antibody. This would need to be confirmed by absorbing the 7C serum with a B27 positive cell. Thus, the use of cell absorption can be very helpful in determining a high PRA patient's antibody as shown in this example.

References

1. Cross DE, Greiner R and Whittier FC: Importance of the autocontrol crossmatch in human renal transplantation. Transplantation 21:307, 1976.
2. Stastny P and Austin CL: Successful kidney transplant in patient with positive crossmatch due to autoantibodies. Transplantation 21:399, 1976.
3. Ting A and Morris PJ: Renal transplantation and B-cell crossmatches with autoantibodies and alloantibodies. The Lancet 2:1095, 1977.
4. Ting A and Morris PJ: Successful transplantation with a positive T and B Cell crossmatch due to autoreactive antibodies. Tissue Antigens 21:219, 1983.
5. Etheredge EE and Anderson CB: Serum autoleukocytotoxic activity and the positive crossmatch in potential allograft recipients. Surgery 83:565, 1978.
6. Connors SM, Myrberg SJ, Dodds K, Carpenter CB and Garovoy MR: Successful renal allograft in the presence of York (Yka) and autologous B-cell antibodies. Transplantation Proceedings XI:1944, 1979.
7. Myrberg SJ, Connors CM, Carpenter CB and Garovoy MR: Positive crossmatches due to autoantibodies in living-related transplantation. Transplantation Proceedings IX:1954, 1979.
8. Braun WE and Zachary AA: The HLA histocompatibility system in autoimmune states. Clinics in Laboratory Medicine 8:351, 1988.
9. Phelan DL, Rodey GE, Flye MW, Hanto DW, Anderson CB and Mohanakumar T: Positive B cell crossmatches: Specificity of antibody and graft outcome. Transplant Proceedings 21:687, 1989.

Extraction of Antibodies from Placentas

Alan R. Smerglia

Principle/Purpose

Placental tissue can provide a valuable source of reagent quality HLA antisera. Procurement of *placentas* should include reliable arrangements for proper handling and transport of these specimens prior to their laboratory processing to insure the biologic and immunologic integrity of each specimen. These protocols should include measures that will preclude cross contamination of placental fluids from multiple specimens procured together, exposure to extreme and/or fluctuating temperatures, and exposure to microbial contamination. Also, if test results for infectious agents are not available for placental specimens upon receipt, a follow-up procedure should be included in sample processing for testing the potential biohazard of those samples which will be handled routinely or exchanged with other laboratories.

Specimen

Aseptic or frozen placental tissue.

Unacceptable specimens are determined to be specimens with improper labeling as established by local protocols; specimens demonstrating observable microbial contamination, i.e., discoloration, odor; and/or specimens positive for infectious agent testing.

Reagents and Supplies

1. 10% stock sodium azide (Na Azide)
2. Dissecting kit including scalpel and scissors
3. Rubber bands
4. Sealable plastic bags

Instrumentation/Special Equipment

1. High speed refrigerated centrifuge
2. Refrigerated area of sufficient size to hang plastic bags containing specimen

Appropriate protective measures (gloves, etc.) must be taken during the processing procedure to prevent exposure to biohazardous material.

Calibration

Not Applicable

Quality Control

Maintenance of a logbook for specimens processed, fluid volumes extracted, and special treatments and/or noteworthy test results obtained will be of use for tracking the handling of these specimens.

Procedure

1. Assign a specimen code number from a logbook for samples to be processed.
2. Transfer placenta from the hospital's container into a dissecting container with the veiny, umbilical cord side facing upward.
3. Cut (cross-section) all major veins visible on the cord side of placenta with scissors or scalpel. In addition, cut away any excess (loose) membrane on the opposite side exposing the spongy tissue beneath to promote drainage of fluid from the placenta.
4. Carefully transfer the placenta and other fluid matter into a clean, labeled and sealable bag (9 x 18 inches) which is constricted in the middle with a rubber band such that the placenta lies cord side down in the upper half of the bag allowing fluid to drain to the bottom half (a hourglass configuration).
5. Remove sufficient air from the bag to allow gentle squeezing of the placenta in a later step and then seal the bag.
6. Fold over the top edge of the bag onto itself and puncture the bag with an opened, S-shaped paper clip hooking the center of the folded bag edge inside the paper clip.

7. Hang the bag securely in a 5° C cold room or refrigerator and allow further drainage of fluid into the bottom of the bag overnight.
8. Retrieve placenta blood the next day as follows:
   a. Squeeze the top half of the bag containing the placenta so that the remaining blood drains to the bottom.
   b. Then carefully cut one bottom corner of bag and pour the fluid into labeled collection tubes.
9. Centrifuge extracted material at 15,000 rpm for 20 min at 5-10° C in a refrigerated centrifuge.
10. Transfer supernatant to clean labeled tubes and repeat step 9.
11. Collect the supernatant into a clean, labeled stock bottle and note, in an appropriate logbook, the total fluid volume collected.
12. Add 1 ml of stock 10% Na Azide solution to every 100 ml of placenta blood collected (to a final 0.1% Na Azide concentration) from each specimen and mark each bottle to identify the addition of the Na Azide.
13. Dispense collected sample into appropriate aliquots for subsequent hepatitis, anti-HIV and/or other infectious agent testing and for HLA antibody screen evaluation as described in Lymphocytotoxic Antibody chapter or by established local procedure.
14. Store aliquots and stock bottle at 5° C awaiting antibody evaluation. **Note:** Inclusion of a freeze/thaw step prior to testing may be advisable for the screen aliquots if antibody positive stock volumes are stored frozen and are to be used or shared without further evaluation.
15. Discard samples that have undesirable antibody screen results and/or positive hepatitis or anti-HIV.
16. Dispense useful stock samples into aliquots for future use and store at -20° C to -70° C.

Calculations

Not Applicable

Results

Not Applicable

Procedure Notes

If multiple samples are collected in a single container the pooled blood may be treated as separate specimen processed as in steps 10 through 16 of the above procedure. Note in logbook those specimens that were processed in this fashion and the identification numbers assigned to the placentas involved.

Reagent quality HLA antibodies can also be obtained from frozen placenta specimens utilizing the described procedure. The volume of blood retrieved from the previously frozen placental tissue will generally be greater probably due to some structural breakdown of internal placental tissue allowing more blood to drain and/or the release of intracellular material from cell lysis. The extracted fluid will be grossly hemolyzed, and this may pose a significant problem of false positives with subsequent enzyme-linked immunosorbent assay (ELISA) based testing for hepatitis and anti-HIV.

Lastly, it may be necessary to carefully remove a light ring of lipid debris (cell membrane fragments) at the top of the centrifuge tube after the first centrifugation step.

Limitations of Procedure

Not Applicable

References

ASHI Quarterly, March, 1983.

Graham ML, Simonis TB, Davey RJ, Harvesting HLA antibodies from placentas. Laboratory Medicine 20:169, 1989.

Immunogenetics and Transplantation Laboratory, The Oregon Health Sciences University (personal communication).

Laboratory Procedure Manual of the Histocompatibility Laboratory, Case Western Reserve University, University Hospitals of Cleveland.

Laboratory Procedure Manual of the HLA Immunogenetics Laboratory, Lombardi Cancer Center, Georgetown University.

Inactivation of IgM Antibodies: A. DTT Treatment and B. Heat Inactivation

Amy B. Hahn

Inactivation of autoantibodies may be critical to the accuracy of crossmatches and antibody screens. Two different methods are presented in this chapter.

Purpose

Antibodies of either the IgG or IgM isotype can bind cells and activate the complement pathway, resulting in cell lysis. IgG antibodies are monomers, the typical Y-shaped molecule with two antigen binding sites. IgM antibodies form pentamers, with five of the Y-shaped molecules crosslinked by disulfide bonds to each other and to a J chain. Because of their pentameric structure, IgM antibodies are very efficient at activating the complement cascade.

Alloantibodies are those antibodies directed against HLA antigens on the cells of others. These are formed in response to sensitization by transplant, transfusion, or pregnancy and are typically (but not always) IgG isotype. Autoantibodies are those directed against self antigens and are typically IgM isotype. In a histocompatibility laboratory, it is not uncommon to have patients whose renal failure was caused by systemic lupus erythematosus. In these patients, the autoantibodies are produced against a variety of antigens composed of nucleic acids and proteins, such as nucleosomes, ribosomes, and small ribonucleoprotein complexes involved in RNA processing.[4] Not only can these autoantibodies bind to a patient's own cells, they can also sometimes bind to the cells of others, cause lysis in the presence of complement, and therefore can be detectable in antibody screens and crossmatches.

In spite of their lymphocytotoxic activity, IgM autoantibodies are not believed to be damaging to transplanted organs. This is in contrast to alloantibodies of the IgM isotype, which may be deleterious to graft survival but to a lesser extent than IgG alloantibodies. An excellent review of the effects of antibodies of different isotypes and specificities is available.[8]

Because autoantibodies are detectable in antibody screens and crossmatches yet irrelevant to solid organ transplant outcome, it is very important to determine if they are present in a patient's serum by autocrossmatching or autoabsorption. If they are present, a technique must be used to distinguish them from IgG alloantibodies. This is typically done by inactivation of the IgM antibodies which, in most clinical laboratories, is achieved by treatment of the serum with DTT or heat.

A. DTT Treatment

Purpose

DTT (dithiothreitol or Cleland's reagent)[2] is a sulfhydryl compound which inactivates IgM antibodies by cleavage of the intersubunit disulphide bonds.[6] IgG antibodies are less susceptible to inactivation by DTT because the disulfide bonds between chains are not as labile as the disulfide bonds between the IgM subunits[5] but they may be slightly affected.[1]

Specimen

Patient peripheral venous blood collected without anticoagulant, such as in a plain red-stoppered vacutainer tube. Allow blood to clot and remove serum to another tube.

1. Specimens that are hemolyzed or do not clot are unacceptable.
2. Before separation of serum from clot, specimens can be stored at room temperature for up to 48 hours or at 4° C for up to one week. Do Not Freeze! After separation, store the serum at -70° C indefinitely.

Reagents and Supplies

1. **DL-Dithiothreitol (Cleland's Reagent, DL-DTT)**
   a. Chemical Formula: $C_4H_{10}O_2S_2$,FW=154.2
   b. Health/Safety Precautions: CAS# 3483-12-3, Route of entry: ingestion, skin or eye contact; Heath Hazard: toxic, irritant; Target Organs: eyes, skin, CNS, mucous membranes; H3 F1 R0.
   c. Source Supply Company: Sigma, St. Louis MO
   d. How to Prepare:
      1) 1M Stock Solution:
         Dissolve 1.543 g DTT in 10 ml Phosphate Buffered Saline (PBS) pH 7.4.

2) 0.05M DTT Working Solution:
Dilute stock solution 1/20 in PBS (1 ml stock plus 19 ml PBS) and pH to 7.0 – 7.5 with 0.5N NaOH. Aliquots of the Working Solution can be made and stored at -70° C for up to one year.

Instrumentation/Special Equipment

37° C heat block, incubator, or water bath

Calibration

Verify that the heat block, incubator, or water bath is 37° C with a thermometer calibrated to one certified by the National Bureau of Standards (NBS).

Quality Control

1. **Dilution Control**
   a. 90 µl of patient serum + 10 µl PBS
   b. Purpose: to show that reactivity was not reduced simply by dilution of serum
   c. Expect: positive if untreated serum is positive
   d. Frequency: in parallel with all samples
2. **Positive Control (IgG)**
   a. high PRA human serum of the IgG isotype, DTT treated and untreated
   b. Purpose: to show that DTT has minimal effect on IgG and is not anti-complementary
   c. Expect: positive treated and untreated
   d. Frequency: at least every new lot of DTT but preferably every antibody screen
3. **IgM Control**
   a. high PRA human serum of the IgM isotype, or IgM anti-human monoclonal antibody, DTT treated and untreated
   b. Purpose: to show that DTT inactivates IgM
   c. Expect: negative treated, positive untreated
   d. Frequency: at least every new lot of DTT but preferably every antibody screen
4. **NegativeControl**
   a. pooled normal human serum, 0% PRA, DTT treated and untreated
   b. Purpose: to show that DTT is not cytotoxic
   c. Expect: negative treated and untreated
   d. Frequency: at least every new lot of DTT but preferably every antibody screen

Procedure

1. Mix 10 µl 0.05M DTT working solution with 90 µl patient's serum (final DTT concentration 0.005M).
2. Mix well and incubate at 37° C for 30 – 45 minutes.
3. Serum is ready for complement dependent cytotoxicity testing in crossmatch or antibody screen

Calculations

Not applicable

Results

Positive crossmatches that are due to antibodies of the IgM isotype should be rendered negative after treatment with DTT. Positive crossmatches that are due to antibodies of the IgG isotype should be unaffected by this treatment.

Procedure Notes

1. DTT inactivation of IgM can be carried out directly in the wells by adding 1 µl of 0.01M DTT to 1 µl serum and incubating for 30 min. at 37° C before the addition of cells.[1]
2. To avoid DTT's anti-complementary effect, 0.002M cystine (DL Cystine, Sigma, St. Louis MO) can be added to the complement[1] or two washes can be performed before adding complement to the wells.[3]
3. DTT treated serum can be frozen and thawed.
4. DTT prepared in phosphate buffered saline will lose reducing ability if stored at 4° C for more than 14 days but will not lose activity if stored frozen at -20° C. Preparation in isotonic saline is recommended if DTT is to be stored at 4° C.[7]
5. DTT inactivation is not normally performed on samples analyzed by ELISA or Flow methodology because those reagents are specific for IgG detection. Special reagents are required to analyze IgM antibodies.

Limitations of Procedure

1. DTT inactivation is not usually performed on sera from bone marrow transplant patients. Most antibody screens performed post -transplant on bone marrow recipients are due to platelet problems. The IgM antibodies found in these patients are usually not autoantibodies. They are true newly formed alloantibodies.
2. DTT can affect the function of complement. To avoid the anti-complementary effect, see Procedure Note #2 above.

References

1. Chapman JR, Taylor CJ, Ting A, and Morris PJ, Immunoglobulin class and specificity of antibodies causing positive T cell crossmatches – relationship to renal transplant outcome. Transplantation 42:608-613, 1986.
2. Cleland WW, Dithiothreitol, a new protective reagent for SH groups. Biol. Chem. 3:480-482, 1964.
3. Fotino M, DTT modification of the lymphocytotoxicity assay, In: *ASHI Laboratory Manual*, 2nd edition, AA Zachary and GA Teresi, eds., American Society for Histocompatibility and Immunogenetics, Lenexa, pp. 321-324, 1990.
4. Janeway CA and Travers P, *Immunobiology: The Immune System in Health and Disease*, 2nd edition, Current Biology Ltd./Garland Publishing Inc., Chap.11, 1996.
5. Moore SB, and Steane EA, Thiol reagents in blood banking. In: *Special Serological Technics Useful in Problem Solving*, RB Dawson, ed,. American Association of Blood Banks, Washington DC, pp. 17-51, 1977.
6. Okuno T, and Kondelis N, Evaluation of dithiothreitol (DTT) for inactivation of IgM antibodies. J. Clin. Path. 31: 1152-1155, 1978.
7. Pirofsky B, and Rosner ER, DTT test: A new method to differentiate IgM and IgG erythrocyte antibodies. Vox Sang. 27:480-488, 1974.
8. Zachary AA, and Hart JM, Relevance of antibody screening and crossmatching in solid organ transplantation. In: *Handbook of Human Immunology*, MS Leffell, NR Rose and AD Donnenberg, eds., CRC Press, Boca Raton; pp. 477-519, 1997.

B. Heat Inactivation

Purpose

The technique of heating serum to remove IgM activity was first mentioned in 1981 by Steinberg and Cook.[1] It has become a popular choice in many laboratories due to advantages of heat inactivation (HI) over DTT: 1. speed; 2. lack of sample dilution; 3. absence of carcinogenic chemicals. Because it works by denaturing heat sensitive proteins, its effects are less specific than DTT. IgG molecules are relatively insensitive to heat and are minimally affected.[2]

Specimen

Same as for DTT procedure.

Reagents and Supplies

No special reagents are needed.

Instrumentation/Special Equipment

1. heat block or water bath
2. microcentrifuge

Calibration

Temperature is critical for this procedure. The heat block or water bath should not vary by more than ± 1 degree. Verify that the heat block or water bath is 63° C with a thermometer calibrated to one certified by the National Bureau of Standards (NBS). For an accurate temperature reading in a heat block, place the thermometer in a tube of water.

Quality Control

1. **Positive Control (IgG)**
   a. high PRA human serum of the IgG isotype, HI treated and untreated
   b. Purpose: to show that HI has minimal effect on IgG and is not anti-complementary
   c. Expect: positive treated and untreated
   d. Frequency: every antibody screen or crossmatch
2. **IgM Control**
   a. high PRA human serum of the IgM isotype, or IgM anti-human monoclonal antibody, HI treated and untreated
   b. Purpose: to show that HI inactivates IgM
   c. Expect: negative treated, positive untreated
   d. Frequency: every antibody screen or crossmatch

3. **Negative Control**
   a. pooled normal human serum, 0% PRA, HI treated and untreated
   b. Purpose: to show that HI is not cytotoxic
   c. Expect: negative treated and untreated
   d. Frequency: every antibody screen or crossmatch

Procedure*

*modification of procedure from Allogen Laboratories (formerly the Cleveland Clinic Foundation Histocompatibility and Immunogenetics Laboratory)

1. Aliquot desired amount of serum into microcentrifuge tube
2. Place serum aliquot in pre-heated 63° C heat block for exactly 13 minutes.
3. Remove from heat block as soon as timer goes off and spin in microcentrifuge for 1 minute.
4. Remove supernatant to another tube labeled to indicate that the sample has been heat inactivated. Be careful not to disturb any pellet or gel that may be in the bottom of the spun tube.
5. Refrigerate or freeze the sample until needed for crossmatch or antibody screen.

Calculations

Not applicable

Results

Positive crossmatches that are due to antibodies of the IgM isotype should be rendered negative after heat inactivation. Positive crossmatches that are due to antibodies of the IgG isotype should be unaffected by this treatment.

Procedure Notes

1. Some samples may form a gel upon heat inactivation. To prevent this, a drop of saline may be added to the serum before heating (approximately 60 µl for 1 ml serum or 12 µl for 200 µl serum). The saline can contain 2% Na azide as an antimicrobial agent.
2. If absorbing the serum with platelets for removal of class I alloantibodies, perform the platelet absorption *before* the heat inactivation. The denatured IgM antibodies may interfere with the platelet absorption.

Limitations of Procedure

1. The effects of heat inactivation are relatively non-specific. In addition to IgM molecules, other heat sensitive proteins may also become denatured. However, for most applications in a Histocompatibility Laboratory, the IgG lymphocytotoxic alloantibodies are the molecules of interest, and they are minimally affected by this procedure.
2. Bone marrow transplant (BMT) patients are the exception. Heat inactivation is not usually performed on BMT recipients. Most antibody screens performed post -transplant on bone marrow recipients are due to platelet problems. The IgM antibodies found in these patients are usually not autoantibodies. They are true newly formed alloantibodies.

References

1. Steinberg AG and Cook CE, *The Distribution of the Human Immunoglobulin Allotypes*. Page 1. Oxford University Press, 1981.
2. Thorne N, Klingman LL, Teresi GA, and Cook DJ: Effects of heat inactivation and DTT treatment of serum on immunoglobulin binding. Hum Immunol 37(supplement 1):123, 1993.

Depletion of OKT3 from Serum

Lori Dombrausky Osowski and Donna Fitzpatrick

Principle

Transplant recipients experiencing acute rejection are often given OKT3 (a murine IgG monoclonal antibody) to help reverse rejection. Serum from patients receiving OKT3 treatment post-transplant may become positive in T cell lymphocytotoxicity assays (e.g., PRA determination or crossmatch).

OKT3 can be removed by absorption with a magnetic bead coated with sheep anti-mouse immunoglobulin. After OKT3 is removed from the serum, any underlying lymphocytotoxic antibody can be detected.

Specimen

Fresh or frozen serum from a patient that has received OKT3 treatment. (The PRA result prior to this procedure would result in an 100% PRA to T cell targets from an OKT3 treated patient.) If a patient has been treated with OKT3, this should be recorded in a permanent patient file.

Unacceptable specimen:
– Specimen more than 72 hrs old
– A hemolyzed serum sample
– Serum that will not clot properly or has fibrin present.

Reagents and Supplies

1. PBS – 1x: To 100 ml 10x stock PBS add dH_2O almost to 1 liter. pH to 7.4, QS to 1 liter. Filter sterilize. Store at 2-6° C. Stability: 6 months.
2. Dynabead M-280 Sheep anti-Mouse IgG (Dynal #112.01) or equivalent solid substrate. Store at 2-6° C. Stability: see expiration date with each lot.
3. Controls
   a. *OKT3 Standard:* Orthoclone OKT3 1mg/ml (Ortho Pharmaceutical Corporation). Store at -80° C. Stability: indefinite.
   b. *Positive control:* OKT3 standard, approximately 1000 ng/ml. Add 10 µl OKT3 Standard to 10 ml PHS (pooled human serum). Store at -80° C . Stability: indefinite.
   c. *Polyvalent antibody control:* This should be a previously tested patient serum with well defined specific antibodies. Store at -80° C. Stability: indefinite.
   d. *Negative control:* PHS. Store at -80° C. Stability: indefinite.

Instrumentation/Special Equipment

1. Dynal Magnet MPC-M
2. 37° C incubator
3. 1.5 ml plastic tubes
4. Automatic pipettor with tips
5. Transfer pipettes
6. 400 microliter tubes

Calibration

If a different substrate is used other than Dynal Beads (as listed under reagents), the appropriate volume to use must be calculated by determining maximum absorption capacity with serial dilutions of the positive control. The volume of the alternate reagent can be substituted at the appropriate steps.

Quality Control

Controls are run with each procedure to assess efficiency of OKT3 depletion.

Procedure

1. Place 4 aliquots of 200 µl Sheep anti-mouse IgG beads into a 1.5 ml plastic tube, using pipet tip.
2. Add approximately 1ml of PBS to tube.
3. Place tubes on magnet for 1 minute. (NOTE: centrifugation can replace this step for a non-magnetic substrate)
4. Remove and discard supernatant, using a transfer pipet.
5. Repeat steps 2, 3, and 4 to complete washing of the beads.
6. Add 0.2ml of the following samples to the four aliquots of beads:
   a. Patient's serum (unknown)
   b. PHS (negative control)
   c. Multispecific PRA patient (polyvalent antibody control)
   d. OKT3 Standard, approximately 1000 ng/ml (positive control)
7. Incubate at 37° C for 45 minutes with intermittent mixing.
8. Place tubes on magnet for 1 minute
9. Remove samples with transfer pipettes and place in four new, labeled 400 ml tubes
10. Store treated samples at -20° C. Samples are now ready for testing.
11. Check all of the samples for the presence of lymphocytotoxic antibody by the methodology which has been established in your laboratory or see Lymphocytotoxic Antibody Screening chapter.
12. Repeat procedure if patient serum is not 0% (see limitations of procedure).

Calculations

Not applicable

Results

Samples	Percent Panel Reactive Antibody (% PRA)	
	Untreated	**Treated**
Positive Control	100%	0%
Negative Control (PHS)	0%	0%
Polyvalent Antibody Control	Known % PRA with known antibody specificity	Same as untreated
Patient Serum	Usually 100%	0%

* The percentage of positive PRA may vary depending upon the true sensitization of patient and amount of OKT3 present in serum. (see limitations of procedure)

If all three controls perform as they should, then the procedure has worked properly.

Procedure Notes

OKT3 is usually given daily for 10-14 days following diagnosis of acute rejections. Trough levels of OKT3 in plasma increase during the first two days of treatment and then reach a steady state of approximately 900 ng/ml for the remainder of the treatment course. OKT3 is cleared from the plasma within 48 to 72 hours after the last dose.

This procedure may also be utilized when screening sera by flow cytometric techniques, if the secondary antibody is crossreactive with the mouse immunoglobulin.

Limitations of Procedure

If the patient serum is not 0% after performing this procedure, then the serum may have contained more than 1000 ng/ml OKT3. The procedure can be repeated to assure complete removal of OKT3. If the reactivity is not removed after double treatment and remains stable, then a true PRA/antibody has been identified.

References

1. Dombrausky L and Nikaein A. Depletion of OKT3 from serum. ASHI Laboratory Manual 3rd ed., I.D.3.1 – I.D.3.3.
2. Goldstein G, Norman D, Henell K, Smith I. Pharmacokinetic study of study of orthoclone OKT3 serum levels during treatment of acute renal allograft rejection. Transplantation 1988; 46:587-589.
3. Written correspondence from John J. Fung, MD, PhD, University of Pittsburgh School of Medicine.

General Concepts in Preparation of Monoclonal Antibodies

Paul J. Martin

With the advent of monoclonal antibodies has come a quantum leap in the analysis of HLA polymorphism. Unlike conventional antisera, the antibody from a hybridoma can be produced in quantities limited only by the availability of culture medium and incubator space or by the number of mice inoculated for ascites generation. Furthermore, monoclonal antibodies offer a reproducibility of specificity unparalleled by polyclonal antisera. This chapter will focus on selected issues concerning the use and maintenance of existing monoclonal antibodies or hybridoma cell lines. Readers interested in specific issues concerning the generation of new monoclonal antibodies are referred to the appropriate chapters of Methods in Enzymology, Volume 121 and the Handbook of Experimental Immunology, Volume 4. These references provide an excellent resource for information about immunization schedules, selection of hybridoma partners, fusion protocols and screening strategies.

General Concepts

Immunization causes a selective proliferation and differentiation of the population of B lymphocytes that produce antibodies which recognize antigen. Conventional human antisera thus contain a mixture of all antibodies produced in response to the immunization that results from transfusion or pregnancy. In this situation, antibodies against nonpolymorphic determinants cannot be produced and these reagents recognize only polymorphic regions of HLA molecules. However, the supply of conventional antisera is limited and even functionally "monospecific" reagents contain multiple antibody species. Most monoclonal antibodies are generated by immunizing a foreign species such as mouse or rat since well characterized myeloma fusion partners are available from these species. Human hybridomas have been produced but are limited by the difficulty in obtaining large numbers of recently boosted immunized cells and by the lack of myeloma partners capable of producing large amounts of immunoglobulin in vitro.[1] The success of interspecies fusion between immunized human cells and mouse or rat myeloma cell lines has been limited by the marked chromosomal instability that often results in loss of antibody production by the hybridoma.[5] Immunization of foreign species such as the mouse or rat with human cells predominantly results in the generation of antibodies against nonpolymorphic determinants. Thus extensive screening may be necessary in order to identify the unusual antibodies that recognize polymorphic determinants.

Hybridomas represent the "machinery" responsible for producing the final antibody product. Fusion between nonimmortalized B cells capable of producing specific antibody and immortalized myeloma cells that produce either no antibody or an antibody of irrelevant specificity results in a hybrid cell line retaining characteristics of both parental cell types: an immortalized cell line capable of producing specific antibody. Fusion mixtures contain many immortalized, antibody-producing hybridomas. If left as a population entirely contained in a single vessel, these cells would produce a complex antibody mixture analogous to that present in the serum of an immunized animal. Whereas antibodies of distinct specificity cannot easily be separated from each other, the immortalized cells that produce the antibodies can be readily separated by a variety of cloning techniques. This results in the isolation of cell populations each derived from a single progenitor.

Fusion of two diploid cells initially results in a tetraploid cell that reverts to near diploid status by random loss of extra chromosomes. Hybridomas continue to produce antibody only if the chromosomes carrying specific immunoglobulin heavy chain and light chain genes are retained. Cloned hybridomas may actually produce more than one antibody species if the myeloma parent contains active immunoglobulin heavy chain or light chain genes.[4] For example, the NS-1 myeloma produces a kappa light chain, and hybridomas made with NS-1 may contain three antibody species: one that contains only light chain from the immune cell parent, one that contains a single light chain from each parent, and one that contains only light chain from the myeloma parent. The first can bind antigen bivalently, the second binds monovalently, whereas the third will show no binding activity. Under ordinary circumstances the myeloma light chain does not affect the specificity of a monoclonal antibody.

Production

Hybridomas derived from myeloma cell lines continuously secrete antibody into their environment as long as the genes necessary for immunoglobulin synthesis are retained by the cells. Spent supernatants from cultures of these cells will typically contain antibody at concentrations of 10-100 mg/ml. Cultures for the generation of antibody can be established in conventional flasks. More recently automated hollow fiber "bioreactors" with continuous replenishment of medium have been developed for large-scale in vitro production of monoclonal antibodies. Alternatively, hybridoma cells can be inoculated intraperitoneally in mice.[3] This often results in the generation of ascites fluid containing antibodies at concentrations of 1-10 mg/ml. Ascites production is facilitated by intraperitoneal administration of pristane 1-2 weeks before inoculation of hybridoma cells. Intraperitoneal growth of hybridoma cells is best accomplished in immunodeficient nude mice or nude rats, although hybridomas can be propagated in immunocompetent hosts if there is MHC com-

patibility. The choice of in vitro or in vivo production depends largely on considerations of the species from which the hybridoma was generated, amount of antibody needed, resources and facilities available, purity required and cost.

Handling and Storage of Cells and Antibodies

A quality control assurance that unrecognized cross-contamination has not occurred represents the most crucial issue for handling and storage of monoclonal antibodies and cell lines. It should be recognized that a single extraneous cell can ultimately overgrow an entire culture and thereby change the antibody being produced. Likewise small amounts of high titer ascites fluid extraneously added to another antibody can give rise to spurious reactivity. This is most often apparent with "negative control" antibodies. Rigorous care must also be taken to prevent misidentification of cells and antibodies. Each ascites fluid considered for inclusion in a pool should be checked by cellulose acetate electrophoresis to demonstrate a paraprotein with mobility identical to that of control. Pooled ascites fluids should show a monophasic titration curve by indirect immunofluorescence assays. Reagents of doubtful identity or quality must be discarded. It is essential that a large number of early passage seed cultures be cryopreserved for each hybridoma. Cells should not be extensively passaged in vitro or in vivo since spontaneous culture changes can occur resulting in selection of variants having reduced antibody production or altered antibody specificity. Alternatively, cultures can be recloned in order to recover sublines having characteristics of the original culture.

As with all serologic reagents, care must be taken to prevent inadvertent bacterial contamination and protease-mediated degradation. This is most easily accomplished by addition of 0.1% azide and storage at 4° C. Culture supernatants and ascites fluids may also be filter sterilized (0.45 μ or 0.22 μ). Freezing at -70° C is recommended for long-term storage of ascites fluid. Carrier proteins such as bovine serum albumin or human serum albumin should be included when using purified antibodies at low concentration in order to prevent nonspecific loss on plastic or glass surfaces.

Purification

Antibodies can be purified from either culture supernatants or ascites fluids by a variety of techniques.[2] The most simple procedures involve ammonium sulfate precipitation for IgG antibodies or dialysis against low ionic strength for IgM antibodies. Better results can be achieved by various chromatographic techniques which remove contaminating proteases. Mouse IgG antibodies are probably most effectively purified by protein A or protein G-Sepharose chromatography. The binding of mouse IgG1 can often be improved by raising the pH. Ion exchange chromatography can also be used to purify either IgG or IgM antibodies. With ion exhange chromatography, antibodies are eluted by increasing the ionic strength. This avoids the potentially denaturing changes in pH necessary for elution from protein A. Either procedure can be adapted for high performance liquid chromatography with significiant gain in speed and efficiency at the expense of increased cost.

It should be recognized that the bovine serum frequently included in culture medium contains immunoglobulin that can copurify with monoclonal antibody. Likewise, ascites fluids contain significant amounts of irrelevant murine immunoglobulin. These antibodies generally will not interfere with most applications unless high specific activity is necessary. In the latter situation, it may be helpful to use antibody produced in vitro, in serum-free medium or in medium containing a reduced concentration of serum. Alternatively, serum can be depleted of antibody by protein A-Sepharose chromatography.

Uses of Monoclonal Antibodies

Monoclonal antibodies have found extensive use in the laboratory and are also beginning to be evaluated as a new therapeutic modality. Antibodies can be employed for conventional serologic analysis by cytotoxicity testing since many murine monoclonal antibodies except those of the IgG1 subclass can mediate complement-dependent cytolysis. Monoclonal antibodies can also be used to assess specificities recognized by proliferative or cytotoxic T cell clones since the binding of antibody can block functional responses. Monoclonal antibodies have been used as a direct probe with which to assess the function of cell surface molecules. For example, the mitogenic activity of certain anti-CD3 antibodies led to the identification of the receptor by which human T cells recognize antigen. As a more practical application in the histocompatibility laboratory, monoclonal antibodies have been extremely useful in the detection of HLA antigens. In addition, monoclonal antibodies can be employed to isolate subsets of lymphoid and hematopoietic cells for serological and functional testing. In the short span of less than 20 years since monoclonal antibodies were first developed, this technology has found wide-spread use and application in clinical laboratories. The trend for increased use of monoclonal reagents is certain to continue in the future.

References

1. Abrams PG, Rossio JL, Stevenson HC, Foon KA: Optimal strategies for developing human-human monoclonal antibodies. Methods in Enzymology 121:107, 1986.
2. Bruck C, Drebin JA, Glineur C, Portetelle D: Purification of mouse monoclonal antibodies from ascitic fluid by DEAE affi-gel blue chromatography. Methods in Enzymology 121:587, 1986.
3. Hoogenraad NJ, Wraight CJ: The effect of pristane on ascites tumor formation and monoclonal antibody production. Methods in Enzymology 121:375, 1986.
4. Milstein C: Overview: Monoclonal antibodies. In: Handbook of Experimental Immunology, vol. 4: Applications of Immunological Methods in Biomedical Sciences; DM Weir, ed.; Blackwell Scientific Publications, Oxford, 1986.
5. Westerwoudt RJ: Factors affecting production of monoclonal antibodies. Methods in Enzymology 121:3, 1986.

The Basic Lymphocyte Microcytotoxicity Tests: Standard and AHG Enhancement

Katherine A. Hopkins

Purpose

The basic lymphocyte microcytotoxicity technique is a consequence of agreement by many investigators with respect to conditions that provide a simple, reproducible and sensitive assay for HLA-A, B, C, and DR antigens on lymphocytes. While molecular techniques overcome the problem of antigen expression problems on the cell membrane, cytotoxic testing remains popular due to its speed, reproducibility, cost, and relatively inexpensive instrumentation requirements. The basis of the procedure is cytolysis mediated by specific antibody in the presence of complement. Sensitizing reagents such as antiglobulin may enhance detection of antibody, but are not used for antigen identification.

The majority of reagents currently in use have been operationally defined, and therefore, this procedure is sensitive to minor alterations in the protocol. All reagents should have been quality controlled, and typing sera should have been selected on the basis of their performance with this technique (see Quality Controls section). Class II antisera should be platelet absorbed to remove contaminating Class I activity.

The sensitivity of this test has been found insufficient to be reliable as a sole crossmatch test without modifications in incubations or additions of wash steps or sensitizing reagents.

Specimen

A lymphocyte suspension prepared by any method that provides a viable sample free of contamination with non-lymphocyte cells (see Cell Isolation chapters). Any specimen with less than 80% viability or excessive contamination with granulocytes, red cells, or platelets is unacceptable. Fluorescent procedures which mark lymphocytes without purification of the cell preparation are also acceptable.

Reagents and Supplies

Liquid Petrolatum

1. Paraffin oil or light mineral oil.
2. Mineral oil is added to microtiter tray wells to protect the antisera in the freezer and prevent evaporation. Any mineral oil can be used as long as it is light, clear and nontoxic to cells.

Eosin Technique

1. Eosin Y
   a. Reagents:
      1) Eosin 1 g
      2) distilled H_2O 19 ml
   b. Dissolve eosin in water.
   c. Filter and store in the dark at 4° C. Check for precipitation which may occur during storage and refilter if necessary.
2. Formaldehyde
   a. Reagents:
      1) reagent grade formaldehyde 500 ml
      2) Phenol red 2 ml
      3) 1N potassium hydroxide (KOH) or sodium hydroxide (NaOH)
   b. Add phenol red to formaldehyde.
   c. Add KOH or NaOH dropwise to achieve pH 7.2-7.4 (salmon color).
   d. Store at room temperature (20-25° C) and adjust pH as necessary.

Trypan Blue Technique

1. Stock solution:
   a. Reagents:
      1) Trypan blue 1 g
      2) distilled H_2O 99 ml
   b. Dissolve trypan blue in water.
   c. Filter, centrifuge and store at 4° C.
2. Working solution:
   a. Reagents:
      1) Trypan blue stock solution 3 ml
      2) EDTA-barbital buffer solution (pH 7.0-7.4) 7 ml
   b. Mix.

Fluorescence Techniques

1. See also chapter on Immunomagnetic Isolation of Lymphocyte Subsets Using Monoclonal Antibody Coated Beads
2. Acridine Orange (AO)/Ethidium Bromide (EB)
   a. Stock solution:
      1) Reagents:
         i. Ethidium bromide 50 mg
         ii. Acridine orange 15 mg
      2) Dissolve in 1 ml of 95% ethanol.
      3) Add 49 ml phosphate buffered saline (PBS).
      4) Mix well.
      5) Divide into 1 ml aliquots and freeze at -70° C.
      6) Store for one year.
   b. Working solution:
      1) Thaw 1 ml aliquot.
      2) Add 10 ml 5% EDTA in PBS-azide.
      3) Store in amber bottle at 2-8° C for up to one month.
3. Quenching solution:
      1) Higgins India Ink 7.5 ml
      2) Add to 100 ml PBS.
      3) Store at 2-8° C for up to six months.
4. Carboxyfluorescein Diacetate (CFDA)/EB
   a. Stock solution--CFDA:
      1) Dissolve 100 mg CFDA in 10 ml acetone in a polypropylene tube.
      2) Freeze in polypropylene tubes in 3 ml aliquots at -20° C.
   b. Working solution--CFDA:
      1) Prepare 1X PBS at pH 5.5 using the pH meter and adding concentrated HCl.
      2) Add 3 ml CFDA stock solution to the 500 ml 1X PBS (pH 5.5).
      3) Store at 4° C in 100ml aliquots in brown bottles.
   c. Stock solution--EB:
      1) Dissolve two tablets (11 mg each) in 2 ml distilled water.
      2) Add 20 ml 1X PBS.
      3) Heat in water bath at 56° C for 30 min.
      4) Store at 4° C in brown bottle.
   d. Working solution–EB:
      1) Add 10 ml stock solution (EB) to 500 ml complement.
      2) Freeze at -70° C.
5. Quenching solution--Hemoglobin:
   a. Reagent Preparation:
      1) EDTA
         i. Prepare 5% EDTA (di-sodium) PBS by dissolving 25 g EDTA in 450 ml PBS. Bring final volume to 500 ml with PBS.
         ii. Using pH meter, pH to 7.2 using NaOH pellets. When you near pH 7.2, use dilute NaOH to reach end point. If you go beyond pH 7.2 you will have a gelatinous mass and will need to start over.
      2) Hemoglobin
         i. Dissolve 50 g hemoglobin in 500 ml 5% EDTA PBS in large beaker with a stir bar. When hemoglobin has gone into solution remove stir bar and
         ii. Add 5 ml 1% sodium azide (see below) and pour into 50 ml aliquots in polypropylene conical tubes.
         iii. Centrifuge at 1000 x g for 45 min.

iv. Decant supernate into 50 ml polypropylene conical tubes. and freeze at -20° C. Discard pellet.

3) Sodium Azide
   i. Preparation of 1% sodium azide
   ii. Dissolve 1 g sodium azide in 100 ml PBS.

Anti-Human Globulin Technique

1. Goat anti-human kappa light chain IgG fraction 2 ml
2. Barbital buffer or PBS 8 ml

Instrumentation/Special Equipment

1. An inverted or upright phase contrast microscope with a 10X objective and 10X or 15X eyepieces (for eosin dye-stained cells).
2. Phase contrast or bright field illumination (for trypan blue-stained cells). Cover glasses should be used for all phase contrast reading.
3. Fluorescence microscope if fluorescence dyes are used. A fluorescent microscope adapted with a xenon or mercury lamp and appropriate band pass exciter/barrier filter is excellent for this purpose.
4. pH meter or pH paper for reagent preparation
5. refrigerator or cold room
6. Centrifuge and rotor capable of attaining specified speeds and g forces. Centrifuge rotors and buckets capable of holding appropriate tube or tray sizes.

Calibration

1. Phase contrast microscopes must be tested regularly by using a centering eyepiece provided by the microscope manufacturer to properly align the phase rings.
2. Standard calibrations for centrifuge rotor speed, all thermometers and temperature regulated equipment, pH meter and microscopes should be performed and must be documented. Centrifuge and rotor should be capable of reaching appropriate speeds and generating appropriate g forces.

Quality Control

Standard reagent and equipment QC procedures should be performed and must be documented. In particular, reagents with specified pH ranges need to be checked.

Procedures

Preparation of Typing Trays

If you use commercially available typing trays, skip to testing steps below.
If commercial typing trays are used, assure that the appropriate measures in manufacture (as described below) are followed.

1. Add 2-5 µl of liquid petrolatum (light weight mineral oil) to each well to retard evaporation.
2. Add 1 µl of typing serum to each well, under the oil.
3. Include known positive and negative sera as controls.
4. Store trays in a -70° C freezer until used. Trays to be stored more than 1 month should be wrapped in cellophane or sealed in airtight containers.

Testing: Eosin

1. Adjust concentration of cell suspension to 2 x 10^6 cells/ml and thoroughly mix.
2. Thaw typing trays immediately before using. Check for empty wells while still frozen.
3. Add 1 µl of thoroughly mixed cell suspension to each well. Drop on top of oil, being careful not to touch serum with the needle tip, to avoid carry over.
4. (If necessary, mix cells and sera thoroughly with a needle. Clean needle between sera.)
5. Incubate at room temperature (20-25° C) for 30 minutes.
6. Add 5 µl of pretested rabbit complement to each well. Mix.
7. Incubate at 20-25° C for 60 minutes.
8. Staining:
   a. Add 2-5 µl of 5% eosin solution to each well. Mix if necessary.
   b. Immediately follow with 5-10 µl of pH-adjusted (pH 7.2-7.4) formaldehyde to each well (enough to make a well-rounded meniscus). Mix if necessary. To expedite reading, allow cells to settle 10 minutes.
   c. Lower a 50 x 75 mm microscope slide onto wells in order to flatten the top of the droplet. Avoid formation of bubbles in the wells. Allow cells to settle for 10 minutes.

d. If not reading immediately, liquid petrolatum may be added around rim of the slide to prevent evaporation and siphoning of fluid from individual wells (this step will also reduce exposure of personnel to formaldehyde fumes).

9. Trays to which formalin has been added can be stored for several days if lidded tightly and kept in the refrigerator. They can also be stored at -20° C for 2-4 weeks. Frozen trays should be read within 1 hour after thawing.

Testing: Trypan Blue

1. Adjust concentration of cell suspension to 2 x 10^6 cells/ml and thoroughly mix.
2. Check viability with trypan blue, hemocytometer and phase contrast microscope.
3. Thaw typing trays immediately before using. Check for empty wells while still frozen.
4. Add 1 µl of thoroughly mixed cell suspension to each well. Drop on top of oil, being careful not to touch serum with the needle tip, to avoid carry over.
5. If necessary, mix cells and sera thoroughly with a needle. Clean needle between sera.
6. Incubate at 20-25° C for 30 minutes.
7. Add 5 µl of pretested rabbit complement to each well. Mix.
8. Incubate at 20-25° C for 60 minutes.
9. Staining:
   a. Remove excess complement by pipetting or by "flicking" or snapping the tray with a quick motion of the wrist, being careful not to cast off the cells.
   b. Fill each well with a 0.3% trypan blue solution being careful not to disrupt cells. Allow to settle for 5-10 min, then "flick" off excess dye as in previous step.
10. Optional: add 5-10 µl of buffer (or other suitable diluent) to each well or fill each well to near overflowing with buffer to flatten the top of the droplet, and add a cover slip. Allow to settle before reading.

Testing: Acridine Orange/Ethidium Bromide (Immunomagnetic Beads)

1. Adjust concentration of cell suspension to 2 x 10^6 cells/ml and thoroughly mix.
2. Thaw typing trays immediately before using. Check for empty wells while still frozen.
3. Add 1 µl of thoroughly mixed cell suspension to each well. Drop on top of oil, being careful not to touch serum with the needle tip, to avoid carry over.
4. If necessary, mix cells and sera thoroughly with a needle. Clean needle between sera.
5. Incubate for 40 minutes at 18-22° C in a dark place.
6. Add 5 µl of pretested rabbit complement to each well. Mix.
7. Incubate for 55 minutes at 18-22° C in a dark place.
8. Staining:
   a. Add 2 µl Acridine Orange/Ethidium Bromide solution to each well; incubate for 15 minutes at 18-22° C in a dark place.
   b. Add 5 µl quenching solution to each well. Allow to settle.

Testing: Carboxyfluorescein Diacetate/Ethidium Bromide (Immunomagnetic Beads)

Use cell isolation technique using immunomagnetic beads. See Immunomagnetic Isolation of Lymphocyte Subsets Using Monoclonal Antibody-Coated Beads in this manual (1.A.5.1).

1. Adjust concentration of cell suspension to 3 x 10^6 cells/ml and check viability, using EB and fluorescent microscope.
2. Thaw typing trays immediately before using. Check for empty wells while still frozen.
3. Add 1 µl of thoroughly mixed cell suspension to each well. Drop on top of oil, being careful not to touch serum with the needle tip, to avoid carry over.
4. If necessary, mix cells and sera thoroughly with a needle. Clean needle between sera.
5. Incubate for 30 minutes at 18-22° C in a dark place.
6. Add 5 µl of complement/EB working solution to each well. Mix.
7. Incubate at 20-25° C for 60 minutes in a dark place.
8. Add 5 µl hemoglobin quenching solution.
9. Read immediately, or store for up to 24 hrs at 4° C.

Testing: Carboxyfluorescein Diacetate/Ethidium Bromide (Ficoll Hypaque)

Use lymphocytes isolated according to standard techniques.

1. Check viability of cell suspension
   a. Adjust concentration to 2-3 x 10^6 cells/ml.
   b. Add 0.5 ml CFDA working solution to cell preparation and gently mix.
   c. Cap tube and incubate 5 minutes at 20-25° C in the dark.
   d. Spin tube at 1000 x g for 1 min, remove and discard supernatant.
   e. Repeat wash step twice.

f. Adjust concentration of cell suspension to 3 x 10^6 cells/ml and check viability, using EB and fluorescent microscope.

2. Thaw typing trays immediately before using. Check for empty wells while still frozen.
3. Add 1 µl of thoroughly mixed cell suspension to each well. Drop on top of oil, being careful not to touch serum with the needle tip, to avoid carry over.
4. If necessary, mix cells and sera thoroughly with a needle. Clean needle between sera.
5. Incubate for 30 minutes at 18-22° C in a dark place.
6. Add 5 µl of complement/EB working solution to each well. Mix.
7. Incubate at 20-25° C for 60 minutes in a dark place.
8. Add 5 µl hemoglobin quenching solution.
9. Read immediately, or store for up to 24 hrs at 4° C.

The Anti-Human Globulin Augmented Cytotoxicity Technique (AHG-CDC)

The antiglobulin variation of the basic microcytotoxicity test is used only for crossmatch and antibody screening. Where antigen-antibody reactions occur, the conformation of the antibody molecule is altered, exposing a site on the Fc portion of the Ig molecule that binds the first component of complement. This conformational change requires that both Fab portions of the antibody molecule are bound to antigen. Complement activation requires binding of complement to 2 Fc regions in close proximity to each other. If there is insufficient antibody, Fc regions of adjacent antibody molecules may be too far apart. Also, not all Ig classes activate complement with equal efficiency. The binding of the anti-globulin to the antibody previously attached to the cellular antigens gives the complement a larger number of binding sites and can enhance the strength of the reactions. This enhancement lowers the amount of antibody necessary in a sample for cytotoxic detection.

Anti-human globulin (AHG) is used in crossmatch or antibody screening assays to enhance the sensitivity, i.e., to detect low levels of anti-HLA antibody and non-complement binding antibodies.

1. Design tray layout and pre-label trays and worksheets.
2. For crossmatch assays, make appropriate dilutions of sera.
3. Add serum (crossmatch) or cells (antibody screening)
   a. For crossmatch, add 1 µl T or B lymphocytes (2.2-3.0 x 10^6/ml) to 1 µl patient serum.
   b. If you use commercially frozen trays for antibody screening, add 1 µl of patient serum to each well.
4. Incubate cells and sera
   a. Incubate 30-60 minutes 4° C, 20-25° C or 37° C for crossmatching
   b. Incubate 30-60 minutes at 20-25° C for antibody screening
5. Wash sensitized cells
   a. Flick off excess serum & oil from wells.
   b. Add 10 µl medium to each well for first wash.
   c. Spin trays at 350 x g for 30 seconds.
6. Repeat steps 5:a-c 2 or 3 times using 5 µl medium (for a total of 3-4 washes).
7. Add 1 µl AHG at appropriate dilution.
8. Incubate 2 minutes at 20-25° C. Incubation time is critical.
9. Add 5 µl complement at appropriate dilution.
10. Incubate 60-90 min, at 20-25° C.
11. Stain with one of the vital dyes described above; assess cell injury using the calculation scale below.

For detailed interpretation of results, see chapter on Interpretation of Crossmatch Tests in this manual.

Calculations

Score reactions by estimating the percent of cell death beyond that of the negative control. Record results according to ASHI Standards and the following scale:

Results

Score	Interpretation	% Dead Cells
1	Negative	0-10
2	Doubtful negative	11-20
4	Weak positive	21-50
6	Positive	51-80
8	Strongly positive	81-100
0	Not readable	

1. This assay distinguishes lymphocytes that exclude (viable) or fail to exclude (non-viable) dyes such as eosin, trypan blue, or ethidium bromide that are able to penetrate cells with damaged membranes. In the fluorescence technique, a dye can also be added to stain intact cell membranes so that viable cells may be seen under florescence.
2. Living cells exclude dye and are small and refractile. Dead lymphocytes contain dye and are larger, flatter, and stained dark. In fluorescence techniques living cells are stained green, dead cells are stained orange.
3. Trays to which formalin has been added can be stored for several days if lidded tightly and kept in the refrigerator. They can also be stored at -20° C for 2-4 weeks. Frozen trays should be read within 1 hr after thawing. Fluorescent staining, if hemoglobin has been used as a quenching reagent, is also stable for several days. Trays with fluorescent staining must be kept in a dark place at 4° C prior to reading.

Procedure Notes:

Variations in the basic microlymphocytotoxicity technique abound, and are primarily due to the universally accepted assumption that the basic lymphocytotoxicity test alone is not sensitive enough for crossmatches, typing of class II antigens, or detecting some class II antibodies. Changes in incubation times, temperatures, different stains and addition of wash steps or enhancing reagents are the primary variations used. In addition, the use of immunomagnetic beads for cell separation has resulted in a variation in cell preparation technique. Each major variation is discussed below.

Variations and Modifications

1. Incubation time
   Test sensitivity can be enhanced by extending the incubation times of the cell/serum incubation step, or the complement incubation step. For DR typing, cells and sera are usually incubated for one hr, and complement for 2 hrs when not using the fluorescence technique (see other chapters on fluorescence).
2. Incubation temperature
   The temperature of incubation of cells and serum can be selected to elicit a desired degree of reactivity in the lymphocytotoxicity test. Temperatures of 4° C, Room temperature 20-25° C, and 37° C are most often used. Generally, 20-25° C is the most commonly used , 37° C is the most sensitive temperature, and 4° C the least sensitive. The temperature at which the complement incubation occurs may also affect sensitivity. This can include incident heating from cells and trays incubating on top of light boxes. All incubations must be in the dark when using fluorescent reagents.
3. Anti-human globulin (AHG)
   See above and chapter on AHG Premixed with Complement: Streamlining of Protocols (1.C.10.1).
4. Staining
   Using various vital stains makes for minor variations in incubation times. Using fluorescent stains requires many of the incubations to be done in the dark to avoid loss of fluorescence, and usually requires the intact cells to be stained a contrasting color, or else under fluorescence only the dead cells can be seen. Reagents and trays containing fluorescent dyes must also be stored in the dark.
5. Wash
   Prior to the addition of complement, excess antibody is washed out of the well by flooding the well with buffer, allowing the cells to settle (or giving the trays a soft spin) and "flicking" the buffer out of the wells. This increases the sensitivity of the test by ridding the well of unbound antibody and anti-complementary factors which can inhibit the test. "Flicking" technique variations are a common source of reaction and result interpretation variations between technologists and among laboratories.
6. Immunomagnetic beads
   Using immunomagnetic beads for cell separation requires some variation in cell concentrations and incubation times. See cell isolation chapters in this manual.
7. Because of the complex nature of the test and the many variations, for ease of use this chapter gives the complete steps and reagent preparation required for each major technique. Minor variations, such as increased incubation times and the addition of antiglobulin, can be inserted into any of these procedures.

Controls

1. Negative Control.
   a. Reagent:
      Most laboratories use fetal calf serum or normal serum from a non-transfused, nulliparous, type AB donor. Both must be screened and found negative for cytotoxic activity and rendered free of complement by heat inactivation. Never use buffer or saline solutions as negative controls, as there must be protein in the well to protect the cells. Without it there will be a 10-20% background of dead cells.
   b. Expected response: negative.
      Negative control is to demonstrate viable cells and ascertain background on individual tray. These wells, for a good scoring, should have a viability of >90%.

2. Positive Control
   a. Reagent:
      The critical factors are that it is complement dependent and provides a strong positive reaction. Some possibilities are: anti-lymphocytic serum, serum from a multi-immunized person or pool of sera from highly immunized individuals, or anti-B2-microglobulin. The reagent should be used at a reaction strength comparable to the sera in the trays, i.e., if most typing sera have titers of 1:2-1:8 and the positive control has a titer of 1:1024, the control should be used at 1:256 or 1:512 to assure that it is comparable to the test sera in its sensitivity to test conditions and complement variability.
   b. Expected response: positive.
      Positive control is to determine that all reagents and procedures required to produce a complement dependent lymphocytotoxic reaction are present.
3. T cell and B cell controls
   a. Reagent:
      Usually monoclonal antibodies to antigens found universally on T or B cells.
   b. Expected response: positive with appropriate subset of cells.
      T and B cell controls are to ascertain the proportion of the cell population of each subtype. In a technique using purified T or B cells, the controls are used to ascertain purity of the cell preparation.

Limitations of Procedure

1. The pre-test viability of the cell suspension is critical to accurate scoring. The membranes of frozen cells, when thawed, can become damaged. Any damage to the cell membrane will allow vital dye to "leak" into the cell and cause a high background or false positive reactions.
2. Prior to the plating of cells, therefore, it is important to test the cells for viability by adding a drop of vital dye to an aliquot of the sample. Dead cells can be removed using a variety of techniques (See chapters on isolation techniques in this manual).
3. Damage of the cell membrane by other mechanisms than the antibody-antigen-complement mechanism can occur at any stage of the test. Toxic substances may include detergents, solvents, microbial products, or any reagent with a pH outside the normal physiologic range. Cells may also die during excessive incubation times. Proper positive and negative controls can assess the extent of this damage (see section, this chapter, on controls).
4. Cell damage can be extensive enough to cause complete lysis, leaving only membrane fragments, or wells or entire trays can be inadvertently missed during plating.
5. Absence of complement-dependent, antibody-mediated cytolysis may be due to any of the following:
   a. Absence of serum or complement.
   b. Inactivation of antibody and/or complement prior to addition to the tray. This generally occurs via any of the known pathways of protein denaturation, aeration, microbial contamination, or precipitation in the presence of high ion concentration.
   c. Improper incubation temperature.
      1) At low temperatures, reaction kinetics are reduced.
      2) At high temperatures, thermolabile components degrade.
   d. Antigen excess caused by an excessive number of cells expressing HLA antigens (usually lymphocytes, platelets, monocytes).
   e. Exposure of cells to a fixative prior to exposure to antibody and complement.
   f. Incomplete staining. This occurs when undiluted complement is not "flicked" out of wells before addition of trypan blue stain. Caused by complement inhibiting staining.
   g. Abbreviated incubation times.

References

1. Amos DB, Bashir H, Boyle W, MacQueen M, and Tillikainen, A simple microcytotoxicity test. Transplantation 7:220, 1969.
2. Dejelo CL, Mogor J, and Zachary AA, DRw typing. In: *AACHT Procedure Manual*, II-3-1, 1981.
3. Dynal Beads (Dynabeads®) Technical Tips manual.
4. Hopkins KA, The basic microlymphocytotoxicity technique. In: *ASHI Laboratory Manual*, 2nd Edition, AA Zachary and GA Teresi, eds., American Society for Histocompatibility and Immunogenetics, Lenexa, 11.1, p 195, l990.
5. Tardif GN, Cytotoxicity testing for HLA-DR. In: *Tissue Typing Reference Manual*, SEOPF, C18, p 1, 1987.
6. Terasaki PI, and McClelland JD, Microdroplet assay of human serum cytotoxins. Nature, pp. 204:998, 1964.
7. van Rood JI, van Leeuwen A, and Ploem JS, Simultaneous detection of two cell populations by two-color fluorescence and application to the recognition of B-cell determinants. Nature, 262:795, 1976.
8. Vyvial, TM, and Kiamar, MS, Cytotoxicity testing for HLA-A,B,C. In: *Tissue Typing Reference Manual*, SEOPF, B16, p 1, 1993.
9. Willoughby PB, Ward FE, and MacQueen JM, Modifications of the microcytotoxicity test. *AACHT Procedure Manual*, II-2-1, 1981.

Serologic Typing of HLA Antigens by Monoclonal Antibodies

Jar-How Lee and Jimmy Loon

Purpose

HLA typing has been made practical by the method of Terasaki and McClelland (known as the NIH Standard method), which uses alloantisera in 1 ml amounts.[4,5] The worldwide exchange of antisera for large-scale testing has allowed the identification of many HLA antigens. This microcytotoxicity testing method is presently the standard testing method for clinical tissue typing.

In 1992, a major modification of the method was published, which allows tissue typing to be done in one step.[2,3] This method is called the *Lambda Monoclonal Tray* (LMT) method and is made possible by using *monoclonal antibodies* (MoAbs). In this method, a MoAb is mixed with an equal volume of complement prior to plating on the tray. The advantages of the LMT method are that a typing can be obtained in 1 hour or less and only 1/10 as much complement is used compared to the NIH method.

The use of conventional alloantisera for tissue typing and characterizing HLA markers is limited by lack of adequate volumes of quality HLA typing reagents, low titer, and rarity of certain specificities. MoAbs fill in for the inadequacies associated with using alloantibodies, because they are high titered and provide an almost endless supply of typing reagents. Some of the rare specificities, such as anti-B46, can be produced in the mouse, eliminating the concerns that xenoimmunization produces antibodies only against the monomorphic determinants of HLA markers.[1] Use of monoclonal reagents avoids the problem of contaminating antibodies in alloantisera, e.g., Class I antisera contaminated with Class II antibodies or B35 antisera containing anti-Cw4. In practice, Class I typing can be obtained from B lymphocytes using the Class I MoAbs. Use of MoAbs also avoids the problem of anti-complement factors found in some alloantisera.

Conventional immunization produces a heterogeneous antibody population of different affinities directed against the epitopes of one or more antigens. The majority of the antibodies have low affinities and bind weakly to their epitopes. Because an alloantiserum contains a pool of these antibodies, effective cytotoxicity results from the combined attachment of the individual antibodies.

A MoAb is a homogeneous antibody population produced from a single hybridoma. In order to make proper use of MoAbs, selection criteria must be established including specificity, strength of reaction, titer, and isotype. A low affinity antibody can rapidly dissociate from the antigen or epitope resulting in false negative reactions. Low affinity results from a not-so-perfect contact surface between the epitope and the antigen binding site of the antibody. In addition to false negatives, this can give rise to false positive reactions with similarly shaped or cross-reacting epitopes. A phenomenon known as heterocliticity may occur in which the antibody raised against an epitope may bind more firmly to some other epitope. The strength index for the false positive reactions may be stronger than the true positive ones, at least theoretically. Heterocliticity is difficult to observe in alloantisera. For tissue typing purposes, high affinity antibodies should be selected to minimize false positive and negative reactions.

The antigen contains a mosaic of epitopes that can elicit the production of antibodies from different B lymphocyte clones. A single epitope may be found on more than one antigen so that a MoAb will behave as duo or multispecific. Epitopes found to be associated with two or more specificities are said to be public epitopes, e.g., A10; whereas those associated with one specificity are called private, e.g., A25. Having amino acid sequence information for the different alleles has helped to explain what appears to be extra or false positive reactions as well as false negative reactions. The specificity of a MoAb may not correlate with that seen with alloantibodies and may detect further heterogeneity in HLA molecules. Identification of epitopes makes it possible to ascribe the corresponding alleles, a task not possible with alloantisera. The ability to identify epitopes make MoAbs a powerful adjunct to DNA typing. Furthermore, for a given allele, the immunologically and, perhaps, clinically important sites can be identified. For example, sites important for the serological determinant and T cell receptor recognition may be detected by MoAbs.

The strength index and reproducibility of the assigned specificities are functions of the affinity and avidity of the antibody for its epitope. Typing method, dye exclusion or fluorescence, incubation conditions, and complement would be expected to have greater affect on the performance of a monoclonal reagent than on an alloantiserum.

Specimen

Blood should be drawn in ACD vacutainers and stored at room temperature (20° – 25° C) until processed. Sodium heparin vacutainers may be used to draw blood if the sample is processed within 24 hours. Blood drawn in vacutainers containing EDTA should be avoided; lithium heparin is unacceptable.

Reagents and Supplies

1. Eosin-Y (sodium base), 5% solution: Filter before each use; store at room temperature.
2. Formaldehyde, 37% reagent grade (formalin): Add 2 ml 5% phenol-red solution to 500 ml formaldehyde; add 10N KOH to adjust pH to 7.2; store at room temperature and filter before each use.
3. Rabbit complement.
4. Carboxyfluorescein diacetate (CFDA)
   a. Stock solution: Dissolve 10 mg CFDA in 1 ml acetone in a glass tube. Store at -20° C in Beckman tubes.
   b. Working solution: Add 30 ml CFDA stock solution to 5 ml PBS at pH 7.2. An alternative solution may be prepared by adding 30 ml CFDA stock solution to 5 ml PBS at pH 5.5. Store at 2 – 5 ° C for up to 1 week.
5. Ethidium bromide (EB) stock solution: Dissolve 50 mg EB in 1 ml distilled water. Add 49 ml PBS. Heat in water bath at 56° C for 30 minutes. Store at -20° C. **CAUTION:** EB is a carcinogen.
6. Ethylenediaminetetraacetic acid (EDTA-disodium salt). 5% solution: Dissolve 5 gm EDTA in 90 ml PBS; adjust pH to 7.2 wth 10M NaOH; bring final volume to 100 ml with PBS.
7. Hemoglobin quench: Dissolve 10 gm lyophilized bovine hemoglobin in 100 ml 5% EDTA PBS; add 1 ml 1% sodium azide; centrifuge 45 minutes at 1000 g; store supernatant at -20° C.
8. Phosphate-buffered saline (PBS)
9. Propidium iodide (PI)

Instrumentation/Special Equipment

N/A

Calibration

N/A

Quality Control

Complement Selection

Selection of complement for the microcytotoxicity test is hampered by lack of high affinity and titer antisera and batch to batch variation of rabbit complement due to heterophile antibodies. Compounding the problem is extra sensitivity of B lymphocytes to rabbit complement heterophile antibodies.

Complement selection and use depend on the test method (dye exclusion or fluorescence), target cell (T or B lymphocytes) and whether immunomagnetic beads are used for cell isolation.

Details of setting up the complement titration test is described in the Complement Quality Control chapter in this manual. Complement samples must be tested against a variety of monoclonal reagents, including broad and narrow specificities as well as weak and strong reagents.

Antibody Titration

To test reagent for use in the NIH Standard method, prepare dilutions of antibodies as one would do for alloantisera. Because MoAbs, particularly ascites fluid, have higher titers than alloantisera, use pipetting devices with disposable tips such as Pipetman, etc.

For use in the LMT Method, prepare dilutions of antibodies and add equal volumes of rabbit complement. It is important to keep the MoAb-Complement mixture cold, 4° C, and to plate the mixture as soon as possible, because complement will weaken over time.

Titration and selection of appropriate complement and antibody dilution is crucial to successful typing by dye exclusion and fluorescence methods. Because the fluorescence methods are more sensitive, careful attention to complement selection is required. Antibody titer differences between dye-exclusion and fluorescence can be as much as 10-fold or more.

Use a reference cell panel to screen and titer complement and to select the proper antibody dilution.

Procedures

NIH Procedure

A detailed description of the basic microcytotoxicity test is presented elsewhere in this manual. In summary, 1 ml MoAb at working dilution is added to each well of a Terasaki tray and stored at -65° C or below until ready to use.

Using “soft-drop” technique:

1. Add to each well 1 µl of a 2 x 10^6/ml suspension of either T or B lymphocytes to the Class I tray or B lymphocytes to the Class II tray.
2. Mix the microdroplets together using an electrostatic mixer. We do not recommend using a piece of wire to mix cells and antibody because of the danger of carry-over from a well containing a high affinity and high titer antibody to the next.

3. Incubate cells and antibody for ½ hour at room temperature.
4. Add 5 µl of rabbit complement and incubate for 1 hour.
5. Stop and fix reactions by adding 5 µl of 5% eosin dye to each well, followed by 5 ml of formaldehyde 2 minutes later. The fluorescence method is described below.

LMT Procedure

In the LMT method, equal volumes of rabbit complement and MoAb at working dilution are premixed; 1 ml is plated onto a Terasaki tray. This method eliminates the complement addition step and also conserves complement. Trays are frozen at -65° C, or below, until ready to use.

Using "soft-drop" technique:

1. Add to each well 1 ml of a 2 x 10^6/ml suspension of either T or B lymphocytes to the Class I tray or B lymphocytes to the Class II tray.
2. Mix the microdroplets together using an electrostatic mixer. Mix the microdroplets together using an electrostatic mixer. We do not recommend using a piece of wire to mix cells and antibody because of the danger of carryover from a well containing a high affinity and high titer antibody to the next.
3. Incubate cells and antibody/complement mixture for 45 – 60 minutes at room temperature.
4. Stop and fix reactions as described above for the NIH method. The exact incubation time used depends on whether the dye-exclusion, fluorescence testing, or immunomagnetic bead method is used.

Fluorescence Testing

Before adding lymphocytes to the tray, add 5 µl CFDA (pH 5.5) to cells; mix and incubate in the dark for 10 minutes at 20 – 25° C. Alternatively, you may add 5 µl of CFDA (pH 7.2) and incubate in the dark for 15 minutes at 37° C. Wash cells twice using PBS and resuspend in McCoy's. At the end of the test, add 10 ml of hemoglobin/EB solution (50 µl of EB stock solution per ml of hemoglobin) per well. PI can be substituted for EB.

Calculations

N/A

Results

The conventional scoring scale is used to record percentage killed. See section on the basic microcytotoxicity test in this manual.

Procedure Notes

Troubleshooting

False positives may be attributed to:

1. Carryover: Check that "soft-drop" method is used to add reagents.
2. Error in diluting antibody resulting in underdilution: Check that correct titration procedures are used including changing pipette tips, adding correct volumes of diluent, etc.
3. Insufficient identification of all possible epitopes recognized
4. Toxic or too strong complement
5. Misinterpretation of titration end point

False negatives may be attributed to:

1. Error in diluting antibody resulting in overdilution
2. Insufficient identification of possible epitopes found only on certain variants
3. Change in pH due to exposure of sera and reagents to CO_2, bacterial contamination
4. Weak complement or inactivated complement in individual wells of LMT
6. Misinterpretation of titration end point

The source of the immunomagnetic beads may require adjustment of incubation times. Check each lot of beads using reference cells. For the NIH method, incubation times for the antibody binding and complement lysis steps may need to be varied.

Limitations of Procedure

Serological typing requires that antigens be expressed; several mutations in alleles have been identified that result in non-expression or low expression of HLA at the cell surface. Cytotoxicity testing requires highly viable cell preparations of 90% or greater and high purity. Contamination by red cells and platelets results in much debris that obscures the lymphocytes and hampers scoring. Granulocytes result in background due to their susceptibility to complement-mediated cell lysis by rabbit heterophile antibodies or may form clumps trapping lymphocytes, giving false negative results. Class II typing requires a B-cell purity of 80% or greater.

References

1. Ferrone S, Dierich MP. *Handbook of Monoclonal Antibodies*. 1985.
2. Lee J-H, Lias M, Loon J, Deng C-T, Etessami S, Chen M, Banh L, Conger N, Connors D, Golding J, Manzo A, Rice M, Soloman E, Tran H and Yang C: A simplified HLA typing procedure by anti-HLA monoclonal antibodies. *Visuals of The Clinical Histocompatibility Workshop*, p 3, 1992a.
3. Lee J-H, Lias M, Loon J, Deng C-T, Etessami S, Chen M, Banh L, Conger N, Connors D, Golding J, Manzo A, Rice M, Soloman E, Tran H and Yang C: One step, one hour HLA typing with monoclonal antibodies. Human Immunology 34 (supplement 1):88, 1992b.
4. Terasaki PI and McClelland JD: Microdroplet assay of human cytotoxins. Nature 204:998, 1964.
5. Terasaki PI, Bernoco D, Park MS, Ozturk G and Iwaki Y: Microdroplet testing for HLA-A, -B, -C, and -D antigens. American Journal of Clinical Pathology 69:103, 1978.

Enhancement of MHC Antigen Expression

Patrick W. Adams and Charles G. Orosz

Purpose

The two types of MHC-encoded gene products which display class I or class II determinants are not equally distributed on somatic cells.[3] Although it is generally stated that class I determinants are displayed by all nucleated cells, there are several exceptions to this rule. In general, all hematopoietic cells display class I MHC-encoded determinants. The distribution of class II determinants is considerably more restricted. Constitutive expression occurs on a relatively small number of cell types, including B lymphocytes and monocytes. However, expression of class II determinants can be induced on additional cell types by exposure to selected lectins and/or lymphokines. The induced expression of class II determinants is frequently accompanied by an increased expression of class I determinants.

The lymphokine, gamma interferon (IFN), is a potent inducer/enhancer of class II MHC antigens on many cell types, including macrophages/monocytes, mast cells, mitogen-simulated T lymphocytes, vascular endothelial cells, fibroblasts, and epithelial cells.[3,10] Human T lymphocytes both produce[9] and respond to[5] IFN following activation with antigens or the lectin, phytohemagglutinin (PHA). Consequently, activated T lymphocytes express high levels of class I and class II MHC-encoded antigens.[2,6] Furthermore, activated T lymphocytes can be propagated for prolonged periods in cultures supplemented with the T cell growth factor, Interleukin 2 (IL-2).[4] Hence large numbers of cells bearing class I and class II antigens can be readily obtained from a small T cell inoculum.

These observations form the basis for the use of activated T lymphocytes for serologic identification of HLA antigens.[1] In general, clinical HLA antigen identification can be complicated by a variety of medical or technical problems. These problems can be circumvented if a small number of T lymphocytes can be obtained from the patient.

Specimen

Anticoagulated blood (sodium heparin or acid citrate dextrose–ACD), 10 ml.

Unacceptable Specimen

Failure to obtain 0.5 x 10^6 viable lymphocytes will render a specimen unacceptable.

Instrumentation

Inverted phase contrast microscope, light or fluorescence

Reagents

Culture Medium

1. RPMI 1640 supplemented with:
   a. 20% pooled human serum
   b. 1.6mM glutamine
   c. 100 units/ml penicillin/streptomycin
   d. 2.38 g/L HEPES buffer
2. PHA
   a. PHA-M
3. IL-2
   a. recombinant human IL-2
4. "Expansion Culture Medium"

a. RPMI 1640 culture medium (with additives)	9.0 ml
b. PHA (final concentration 0.5% vol/vol)	0.05 ml
c. IL-2 (final concentration 100 u/ml)	1.0 ml
	10.05 ml

Procedure

This procedure is performed in two phases. The first phase (steps 1-5) involves the lectin-induced polyclonal activation of T lymphocytes present in a mononuclear cell preparation. The second phase, (steps 6-12) involves the lymphokine-induced differentiation and proliferation of the activated T lymphocytes. This treatment provides cells that are suitable for serologic HLA analysis by standard microcytotoxicity testing.

1. Obtain sterile peripheral blood mononuclear cells (PBMCs). The routine clinical technique of density-dependent cell separation Ficoll-Hypaque (FH) gradients is suitable for this purpose.
2. Suspend 10 x 10^6 PBMCs in 10 ml of culture medium.
3. Add PHA to the lymphocyte suspension to a final concentration of 2.0% PHA (0.2 ml stock PHA in 10 ml culture medium).
4. Incubate tissue culture flasks in upright position for 48 hrs at 37° C in a humidified 5% CO_2 atmosphere.
5. Count the PHA-activated lymphoblasts as follows.
   a. Agitate the flasks to resuspend the lymphocytes.
   b. Sample the lymphocyte suspension and determine cell number using routine cell counting techniques. Count only large viable blastoid cells. The PHA-stimulated cell populations usually contain 90-100% lymphoblasts.
6. Centrifuge the cells at 300 x g for 10 min.
7. Resuspend the PHA-induced lymphoblasts in "expansion culture medium" to a final concentration of 2 x 10^5 cells/ml.
8. Distribute the lymphoblast suspension into 16 mm tissue culture wells (2.0 ml cell suspension/well).
9. Incubate at 37° C in humidified 5% CO_2 atmosphere.
10. Monitor lymphocyte cultures for cell growth and viability approximately every third day. To do this, carefully mix culture contents to resuspend the lymphocytes, avoiding culture contamination. Sample lymphocyte suspension to determine cell number and viability using routine cell counting techniques.
11. When the cell concentration reaches 1.5-2.0 x 10^6 cells/ml, subculture the lymphocytes in additional 16 mm culture wells (2.0 ml/well) at a cell concentration of 2-3 x 10^5 cells/ml; use the "expansion culture medium" as subculture diluent.
12. By the 8th-10th day after PHA activation the expanded T cell cultures should be suitable for HLA typing using standard microcytotoxicity procedures. HLA-DR expression tends to improve with the length of the lymphocyte culture period. HLA typing is best performed with "B cell complement."

Procedure Notes

1. There will be many times when it is not possible to obtain 10 x 10^6 PBMC for the initial activation with PHA. As few as 5 x 10^5 cells can be used for this procedure.
2. Minor to moderate red blood cell contamination of the PBMC population does not influence this procedure. The red blood cells will eventually be lost by dilution as the activated T cells are subcultured with IL-2 during the second phase of the procedure.
3. Treat the PHA-activated lymphoblasts gently. When necessary, centrifuge lymphocytes only once (10 min, 300 x g) and disperse the cell pellet by gentle hand "flicking."
4. Slow growth of activated T cells in expansion cultures is frequently encountered. If the lymphocytes have not reached a concentration of 2 x 10^6 cells/ml after 4 days of incubation, do not subculture. Rather, wash the cells 1X by centrifugation (10 min, 300 x g) and reculture at 2-3 x 10^5 cells/ml in "fresh expansion culture media." If cells remain inactive after this maneuver, go back to step 3 and reculture the washed lymphocytes with 2% PHA. In this situation HLA-DR typing can be attempted on the third day after the activated lymphocytes have been transferred to expansion cultures.
5. The appearance of the lymphocyte cultures provides a valuable hint as to subculture timing the readiness for HLA analyses. Under ideal conditions, expect the following:
   PHA activation: Blast cells will appear large with irregular surfaces and dense nuclear material. Clumping will be noted, but there should also be free cells. Culture media will turn from orange to yellow.
   IL-2 expansion: Cells will take on a more rounded shape. Few cell clusters, if any, will be noted. The cells will be larger than resting lymphocytes, and retain the physical characteristics of lymphoblasts.
6. There is flexibility in timing of the two culture phases. The initial activation with PHA can be extended to 4 days. Hence, it is not necessary to manipulate the cultures during the weekend. Likewise, the expansion cultures can be monitored for cell growth at times other than every third day. Utilize common sense and tissue culture experience.
7. Alternate method for T cell activation can be used. It has been demonstrated that a combination of PHA (10 mg/ml) and IL-2 (100 u/ml) can induce adequate HLA-DR expression in 3-5 days for successful DR typing.[7]
8. Single color fluorescence is more sensitive than vital dye staining.

Interpretation

Same standards apply as for any serologic determination of HLA-DR.

References

1. Adams PW, Ferguson RM, Vaidya S, Orosz CG: Clinical utility of serologic HLA-DR antigen identification using activated T lymphocytes. Human Immunol 16:295, 1986.
2. Evans RL, Faldetta TJ, Humphreys RE, Pratt DM, Yunis EJ, Schlossman SF: Peripheral human T cells sensitized in mixed leukocyte culture synthesize and express Ia-like antigens. J Exp Med 148:1440, 1978.
3. Halloran PF, Wadgymar A, Autenreid P: The regulation of expression of major histocompatibility complex products. Transplantation 41:413, 1986.
4. Mayer T, Fuller A, Fuller T, Lazarovits A,Boyle L, Kurnick J: Characterization of in vivo-activated allospecific T lymphocytes propagated from human renal allograft biopsies undergoing rejection. J Immunol 134:258, 1985.
5. Miyawaki T, Seki H, Taga K, Taniguchi N: Interferon gamma can augment expression ability of HLA-DR antigens on pokeweed mitogen-stimulated human T lymphocytes. Cell Immunol 89:300, 1984.
6. Nunez G, Giles RC, Ball EJ, Hurley CK, Capra JD, Stastny P: Expression of HLA-DR, MB, MT,and SB antigens on human mononuclear cells: identification of two phenotypically distinct monocyte populations. J Immunol 133:1300, 1984.
7. Owens D, Stempora L, Bray J, Rodey G, Bray, R: A three day T cell activation method for HLA Class II Typing. ASHI Annual Meeting Abstracts, Human Immunology 61, 1990.
8. Reinherz EL, Kung PC, Pesando JM, Ritz J, Goldstein G, Schlossman SR: Ia determinants on human T cell subsets defined by monoclonal antibody activation stimuli required for expression. J Exp Med 150:1472, 1979.
9. Sandvig S, Laskay T, Anderson J, DeLey M, Anderson V: Gamma-interferon is produced by $CD3^+$ and $CD3^-$ lymphocytes. Immunol Rev 97:51, 1987.
10. Trinchieri G, Perussia B: Immune interferon: a pleiotropic lymphokine with multiple effects. Immunol Today 6:131, 1985.
11. Zier KS, Zmijewski CM: The serological definition of polymorphic HLA-D region gene products on cultured T cells. Detection of DR and MT antigens. Transplantation 37:514, 1984.

Granulocyte Antigens and Antibodies

Mary E. Clay, Gail Eiber and Agustin P. Dalmasso

Purpose

Granulocyte antigens and antibodies have been implicated in the pathophysiology of several clinical conditions. These include alloimmune neonatal neutropenia, autoimmune neutropenia, febrile transfusion reactions, severe pulmonary transfusion reactions, drug-induced neutropenia, failure of effective granulocyte transfusion, and neutropenias secondary to many other diseases. Initial granulocyte serology studies were hampered by the presence of contaminating red cells, platelets and lymphocytes in the test systems. The introduction of density gradients for the isolation of pure granulocyte suspensions was a major contribution to the development of granulocyte serology. In addition, other significant advances have been made that contributed to solving the technical and practical problems associated with granulocyte serology studies.

The antigens on the surface of granulocytes may be considered in two general categories: those shared with other tissues and those found only on granulocytes. The first category includes antigens such as I, Ge, Kx, 5a, 5b, Mart and HLA. Of these antigens, HLA poses the major problem for the investigator working with specimens that may contain both granulocyte- and HLA-specific antibodies. To establish the presence of granulocyte-specific antibodies, such specimens require platelet adsorption or testing with the monoclonal antibody immobilization of granulocyte antigens (MAIGA) assay. Although the presence of ABH antigens on granulocytes has been a controversial issue, recent studies have shown that these antigens are not present on granulocytes.

The granulocyte-specific antigens have been identified mainly through studies of alloimmune neonatal neutropenia (i.e., the neutrophil analog of Rh hemolytic disease of the newborn) and autoimmune neutropenia. Through family studies of such cases, a system of granulocyte-specific antigens has been defined. These antigens segregate independently from known red blood cell, platelet-specific and HLA antigens. Historically the nomenclature for the antigen system has been as follows: N designating neutrophil specificity, letters of the alphabet designating different loci, and Arabic numbers designating alleles at each locus. Presently this group is composed of the following antigens: NA1, NA2, NB1, and NB2. However, during the past few years new antigens have been identified that do not fit the criteria of the original system. Currently a new nomenclature system is being developed that allows for designation of newly discovered mutations according to the internationally accepted conventional nomenclature for genetic variants of human proteins.

The NA system antigens (NA1 and NA2) have been extensively investigated and are located on IgG Fc receptor type IIIb (FcgRIIIb; CD16). NB1 is located on a 58-64 kDa glycoprotein on granulocyte surface plasma membranes and intracellularly on the membranes of specific or secondary granules. The molecule on which NB2 is located has not yet been identified. Recently, a new alloantigen, termed SH, on FcgRIIIb was identified and shown to be due to a point mutation in the NA2-FcgRIIIB gene. The reader is referred to references listed at the end of this section for a more comprehensive review of the molecular biology of the currently known granulocyte antigens.

Granulocyte-specific antigens have been detected primarily by agglutination and immunofluorescence techniques. All the above granulocyte-specific antigens are detectable by both assays except for NB2(9a) which cannot be detected by immunofluorescence. Other granulocyte serology methods such as granulocytotoxicity or antibody dependent cell-mediated cytotoxicity do not give reaction patterns showing the same granulocyte antigen specificities. In studies using some of these other assays sera have been identified that appear to define other systems of granulocyte antigens.

Since the presently known granulocyte antigen systems have been "serologically defined" using different assays and, in general, the assays have different sensitivity, the successful detection of granulocyte antibodies often requires that a combination of methods be used. Since this is usually not feasible, the more practical approach is to use one or two basic screening methods and/or refer specimens to specialized laboratories for initial or more extensive evaluation.

A more detailed description of the material presented in this chapter and a comprehensive review of the clinical and laboratory aspects associated with granulocyte serology can be found in: McCullough J, Clay M, Press C, Kline W: *Granulocyte Serology: A Clinical and Laboratory Guide*. American Society of Clinical Pathology, 1988.

Single-Step Separation of Granulocytes on Density Gradients (Granulocyte Isolation Procedure)

Purpose

To isolate granulocytes for use in direct and indirect granulocyte antibody assays and in granulocyte antigen typing.

Specimen

EDTA or ACD anticoagulated blood

Unacceptable Specimen

Clotted, frozen or hemolyzed specimen

Instrumentation

1. Refrigerated centrifuge with a horizontal rotor

Reagents

1. Ficoll-Hypaque upper gradient solution I (specific gravity = 1.077)
   a. 33.9% Hypaque (Nycomed Inc., Princton, NJ) 30 ml
   b. 9% Ficoll (Sigma Chemical Co., St. Louis, MO) 72 ml
   Store at 4° C.
2. Ficoll-Hypaque lower gradient solution II (specific gravity = 1.119)
   a. 50% Hypaque 33 ml
   b. 9% Ficoll 66 ml
   Store at 4° C.

Solutions I and II can be purchased as a commercially prepared separation medium – Mono-PolyTM Resolving medium (ICN Pharmaceuticals, Irvine, CA).

3. 1% Methyl cellulose
   a. 0.9% saline solution 500 ml
   b. methyl cellulose 5 g

Allow methyl cellulose to completely dissolve at room temperature, stirring. Store at 4° C.

Calibration

Each six months preventive maintenance is performed on the centrifuge, the timing is checked and the speed verified with an optical tacometer.

Quality Control

Three different donor specimens are run to check the performance of the new gradients.

Procedure

1. Mix 14 ml anticoagulated blood with 2.5 ml of 1% methyl cellulose in a 16 x 150 mm culture tube. Slant the tube at a 30° angle and allow the red cells to sediment for 20 min at room temperature (RT), leaving a leukocyte-rich plasma (LRP) (exceeding 30 min of sedimentation will result in a reduced white cell yield).
2. Prepare the gradient tubes while the red cells are sedimenting. Place 3 ml of Solution II in a 15 ml conical plastic centrifuge tube and gently lay 3 ml of Solution I onto Solution II. The two solutions must be at RT.
   Alternative technique: First place 3 ml of Solution I into the centrifuge tube. Fill a syringe fitted with a 3.5 inch, 17 gauge spinal needle with Solution II. Placing the needle tip at the bottom of the centrifuge tube, underlayer 3 ml of Solution II.
3. Carefully layer the LRP above the separation gradient. 9 ml of supernatant can be layered onto a gradient tube containing 3 ml Solution II and 3 ml Solution.
4. Spin the tubes in a refrigerated centrifuge with a horizontal rotor at 1650 x g for 15 min at 18° C. After centrifugation three distinct layers are observed:

   Layer 1 (plasma – Solution I interface) consists of mononuclear cells and platelets

   Layer 2 (Solution I-II interface) consists of polymorphonuclear (PMN) cells

   Layer 3 (pellet) consists of red blood cells (RBCs)

5. Wash leukocytes isolated from layer 1 or layer 2 twice with 10 ml of a balanced salt solution or a phosphate buffered saline solution prior to use in any leukocyte assay.

Interpretation

When good separation occurs three distinct layers are produced as described in Step 4, above.

Troubleshooting

In order to insure good separation, on test days each lot of gradient solutions should be tested with blood from 3 normal donors. If three distinct layers are not observed in Step 4, the following adjustments may be made to the gradient solutions.

Problem	Solution
Seepage of mononuclear cells into Solution I (not a distinct layer)	Add approximately 5 ml of 33.9% Hypaque to each 200 ml of solution I; Retest.
Seepage of granulocytes into Solution II (not a distinct layer)	Add approximately 5 ml of 50% Hypaque to each 200 ml of solution II; Retest.
Granulocytes pelleting with RBCs	Add approximately 10 ml of 50% Hypaque to each 200 ml of Solution II; Retest.
Red cells contaminating granulocyte (PMN) layer	Add approximately 5 ml of 9% Ficoll to each 200 ml of solution II; Retest.

Granulocyte Agglutination Assay (Micromethod)

Purpose

For the detection and identification of granulocyte-specific antigens and antibodies. Certain granulocyte antigens and antibodies are detected only by this technology.

Specimen

Serum or plasma

Unacceptable Specimen

Grossly hemolyzed serum or plasma

Instrumentation

1. Refrigerated centrifuge with horizontal rotor
2. Microcentrifuge
3. Dry air incubator
4. Inverted phase microscope. Objective: 6 x dry.

Reagents

1. Phosphate-buffered saline (PBS), pH 7.2
   a. Sodium chloride (NaCl) 8 g
   b. Potassium chloride (KCl) 0.2 g
   c. Sodium phosphate (Na_2HPO_4) 1.15 g
   d. Potassium phosphate (KH_2PO_4) 0.2 g

   Place in volumetric flask and add sterile water until final volume of 1000 ml is reached. Adjust pH to 7.15-7.25. Store at 4-6° C.
2. Bovine serum albumin-ethylenediaminetetraacetic acid solution (BSA-EDTA): PBS containing 3% BSA and 0.4% EDTA, pH 7.2.
3. Granulocyte resuspension solution (GRS): PBS containing 0.4% BSA and 0.5% EDTA.
4. 3.6% NaCl for hypotonic lysis.

Calibration

The temperature of the incubator is recorded on the day of testing. Each six months preventive maintenance is performed on the centrifuge, the timing is checked and the speed is controlled with an optical tacometer. Preventive maintenance is performed yearly on the microscope. For air displacement, multi-setting pipettes, preventive maintenance and gravimetric analysis is performed each six months.

Quality Control

Known positive antiserum (anti-Mart) is run as a positive control. Male AB plasma that has been tested and shown to be negative for neutrophil antibodies, is run as the negative control.

Frequency: The positive and negative controls are run for each donor cell on each day of testing.

Tolerance: The positive control must be ≥1+ agglutination and the negative control must be negative for agglutination.

Corrective Action: If the tolerance is not met, the testing is invalidated for that donor cell and testing is repeated on a later date.

Records: Results are recorded on a worksheet where the agglutination is scored from negative to 4+. The worksheets are stored for 5 years. A panel sheet is developed for each patient from the work sheet. This panel sheet becomes part of the patient file which is stored as paper for 2 years and then copied on to microfilm

Cell Donors

A panel of 5 donors typed for granulocyte antigens is used to screen for granulocyte antibodies.

Procedure

1. Isolate granulocytes as follows:
   a. Follow the granulocyte isolation procedure (above) through Step 4 using EDTA anticoagulated blood.
   b. For preparation of the test granulocytes, modify Step 5 of the granulocyte isolation procedure as follows:
      i. Discard the plasma supernatant and cells from layer 1. Remove the PMN layer (layer 2) and place in a 17 x 100 mm polystyrene culture tube.
      ii. Wash the cells twice with 10 ml PBS. For each wash, centrifuge the tube at 300 x g for 2 min in a refrigerated centrifuge at 17-19° C. Between washings, gently resuspend the cell pellet with the use of a precision pipette and disposable tip, avoiding bubbles. If the cell pellet appears pink [red blood cells (RBC) present], lyse RBC (see "Troubleshooting" section below). If cells are white, proceed to next step.
      iii. After the second wash, gently resuspend the cell pellet and transfer the cells to a Fisher tube. Wash cells once with 1 ml of BSA-EDTA solution at 800 x g for 45 seconds. The cells must be kept in this solution if they are not used immediately.
      iv. Gently resuspend the cells in 1 ml GRS.
      v. Count cells and adjust concentration to 5 x 10^6/ml in GRS.
2. Place 15 ml of mineral oil in the appropriate wells of a 96-well round-bottom microtiter plate.
3. Place 3 ml of each test serum or a selected serum dilution under the oil in the middle of each well.
4. Add 1 ml of the cell suspension to the bottom of each well. Wipe tip and expel 1 drop of cells between different antisera.
5. Incubate the trays at 29-31° C in a dry air incubator.
6. Evaluate the results after 4½ to 6 hrs of incubation on an inverted phase microscope.
7. Results are recorded on a worksheet listing all the specimens tested.
8. Results are transferred from the worksheet to a panel sheet listing the granulocyte typing of each donor for each specimen tested.

Interpretation

The strength of the reaction is graded from 0 to 4+ according to the proportion of cells participating in the reaction.

Percentage of Agglutinated Cells	**Grade**
greater than 90%	4+
50% to 89%	3+
25% to 49%	2+
less than 25%	1+
none	0

Troubleshooting

Hypotonic lysis – to remove contaminating RBC:

Add 3 ml of distilled water to resuspended button of cells in a 17 mm x 100 mm tube. Wait 12 seconds then add 1 ml of 3.6% NaCl. Mix. Fill the rest of the tube with PBS and wash once. There should be no intact RBC visible with the pelleted granulocytes.

Limitation of Procedure

HLA antibodies and IVIG are known to cause non-specific agglutination.

Indirect Granulocyte Immunofluorescence Assay (Micromethod)

Purpose

For the detection and identification of granulocyte specific antigens and antibodies. Certain granulocyte antigens and antibodies are detected only by this method.

Specimen

Serum or plasma

Unacceptable Specimen

Grossly hemolyzed serum or plasma

Instrumentation

1. Jet pipet with 8-needle stream splitter
2. Refrigerated centrifuge with horizontal rotor
3. Dry air incubator
4. Reflected light fluorescence microscope with FITC filtration system (excitation filter: 450-490 nm; dichroic mirror: 510 nm; barrier filter: 515 nm). Objective: 40X dry. NA.0.75.

Reagents

1. Phosphate-buffered saline (PBS) pH 7.0
   a. NaCl 8.2 g
   b. NaH_2PO_4-H_2O 0.142 g
   c. Na_2HPO_4 1.380 g

   Dissolve to 1000 ml sterile water. Adjust pH to 6.95-7.05. Store at 4-6° C.
2. PBS with 0.2% BSA (PBS-BSA)
3. 1% paraformaldehyde
4. Fluorescein-conjugated antihuman immunoglobulin (FITC-AHIg) – $F(ab')_2$ fragments

Calibration

The temperature of the incubator is recorded on the day of testing. Each six months preventive maintenance is performed on the centrifuges, the timing is checked and the speed is controlled with an optical tacometer. Preventive maintenance is performed yearly on the microscope. For air-displacement, multi-setting pipettes, preventive maintenance and gravimetric analysis is performed every six months.

Quality Control

Two known positive antisera (anti-NA1 plus an anti-NA2 and an anti-Mart) are run as positive controls. Male AB plasma that has been tested and shown to be negative for neutrophil antibodies is run as the negative control.

Frequency:	The positive and negative controls are run for each donor cell on each day of testing.
Tolerance:	The positive control must be ≥ 2+ for immunofluorescence and the negative control must be ≤ 1+.
Corrective Action:	If the tolerance is not met, the testing is invalidated for that donor cell and testing is repeated on a later date.

Records: Results are recorded on a worksheet where the fluorescence is scored from negative to 4+. The worksheets are stored as paper for 5 years. A panel sheet is developed for each patient from the worksheet. This panel sheet becomes part of the patient file which is stored as paper for 2 years then copied on to microfilm.

Cell Donors

A panel of 5 donors typed for granulocyte antigens is used to screen for granulocyte antibodies.

Procedure

1. Isolate granulocytes as follows:
   a. Follow the granulocyte isolation procedure using EDTA anticoagulated blood through Step 4.
   b. For the preparation of the test granulocytes, modify Step 5 of the granulocyte isolation procedure as follows:
      i. Discard the plasma supernatant and cells from layer 1. Remove the PMN layer (layer 2) and place in a 17 x 100 mm polystyrene culture tube.
      ii. Wash the cells twice with 10 ml of PBS-BSA, centrifuging at 300 x g for 2 min each time. Between washes, gently resuspend the cell button by manually rocking the tube.
      iii. Add 2 ml of 1% paraformaldehyde to the resuspended cells and mix gently. Incubate for 4 min at RT.
      iv. Wash cells twice more as above (see Step ii).
      v. Gently resuspend the cells in PBS-BSA, adjusting concentration to 10-12 x 10^6/ml.
2. Place 20 ml of each test serum or a selected serum dilution into wells of a U-bottom microtiter plate.
3. Add 20 ml of the cell suspension to each well containing serum. Gently tap plates on a flat surface to mix cells and sera.
4. Cover the plate with a sealer and incubate for 30 min at 36-38° C in a dry air incubator.
5. Wash cells three times. For each wash, add 200 ml PBS-BSA to each well, using a Jet Pipet with stream splitter (caution in needle-positioning is required to avoid cross-contamination of wells). Centrifuge for 1 min at 200 x g, and decant supernatant by vigorously flicking plates. While inverted, blot plates on absorbent gauze.
6. To each well, add 20 ml of an appropriate dilution of FITC-AHIg (see "Troubleshooting" below). Mix by gently rocking plates.
7. Incubate plates for 30 min at 20-24° C in the dark.
8. Wash cells three times, as in Step 5 above.
9. Add 10 ml of glycerol-PBS (3:1) to each well.
10. Using a pipette, gently resuspend cells. Transfer 2-3 ml cell suspension to a clean printed microscope slide. Apply a coverslip, and allow cells to settle in the dark for a minimum of 15 min.
11. Slides are read for fluorescence and the results are recorded on a worksheet listing all the specimens tested.
12. Results are transferred from the worksheet to a panel sheet which lists the granulocyte typing of each donor for each specimen tested.

Interpretation

Slides are read using a fluorescence microscope and the strength of the reaction is graded from 0 to 4+ according to the characteristics of fluorescence staining.

Interpretation of Results

Grade	Characteristics
0	No cell-bound fluorescence
±	Minimal cell-bound fluorescence
1	Fluorescence dots just outline cell membrane distinctly
2	Fluorescence appears as closely-spaced but distinct dots on cell membrane
3	Fluorescence dots on cell membrane are partially merged to form bands
4	Fluorescence appears as solid ring around cell

Troubleshooting

The appropriate dilution of FITC-AHIg is predetermined by checkerboard titrations. The dilution should allow maximal specific fluorescence with minimal background fluorescence. Each new lot number of conjugate is titrated to determine optimal fluorescence. This is done, using dilutions (i.e., 1:1, 1:5, 1:10, etc.) of each of a known positive and negative serum against dilutions (i.e., 1:50, 1:75, 1:100, 1:125, 1:150, etc.) of antiglobulin serum. The dilution of antiglobulin serum is selected which yields the strongest fluorescence with the positive serum, while showing no fluorescence with the negative serum.

Limitation of Procedure

HLA antibodies and IVIG are known to cause nonspecific immunofluorescence.
Monoclonal Antibody Immobilization of Granulocyte Antigens

Monoclonal Antibody Immobilization of Granulocyte Antigens (MAIGA)

Purpose

To provide a procedure for an antigen capture assay which differentiates between neutrophil specific and HLA antibodies detected in the Granulocyte Agglutination and Granulocyte Immunofluorescence assays and identifies multiple granulocyte specific antibodies.

Specimen

Serum or plasma

Unacceptable Specimen

Grossly hemolyzed serum or plasma

Instrumentation

1. 8-channel, fixed volume pipetter for 200 ml, 100 ml, and 50 ml
2. Microplate reader with 405 nm interference filter
3. Refrigerated centrifuge with horizontal rotor
4. Dry air incubator
5. Refrigerated microcentrifuge

Reagents

1. Tris [Tris(hydroxymethyl) aminomethane]
2. NaCl
3. Triton X-100
4. Tween 20
5. $CaCl \times 2H_2O$
6. Na_2CO_3
7. $NaHCO_3$
8. NaN_3
9. p-Nitrophenyl phosphate
10. Diethanolamine
11. $MgCl_2 \times 6H_2O$
12. NaOH
13. Alkaline phosphatase labeled anti-human IgG
14. 22% Bovine serum albumin
15. Monoclonal antibodies for CD16, CD18 and NB1
16. Anti-mouse polyclonal antibody

Calibration

The temperature of the incubator is recorded on the day of testing. Each six months preventive maintenance is performed on the centrifuges, the timing is checked and the speed is verified with an optical tacometer. The temperature of the 4° C refrigerator is continuously monitored by an electronic system. For air displacement, multi-setting pipettes, preventive maintenance and gravimetric analysis is performed each six months.

Quality Control

Known positive antiserum is run as a positive control. Male AB plasma that has been tested and shown to be negative for neutrophil antibodies is run as the negative control.

Frequency: The positive and negative controls are run for each donor cell with each monoclonal antibody on each day of testing.

Tolerance: The positive control must yield an 0.D. that is at least 0.250 and three times that of the negative control. Negative control should be ≤ 0.200 O.D. Readings for duplicate tests should fall within 20% of the mean of the two values.

Corrective Action: If the tolerance is not met, the testing is invalidated for that donor cell and testing is repeated. If duplicate readings of tests fall outside 20% of the mean or one reading is positive and the other is negative, testing should be repeated.

Records: The absorbance for each specimen with each cell and monoclonal antibody is recorded on a worksheet. The worksheets are stored as paper for 5 years. A panel sheet is developed for each patient from the worksheet. This panel sheet becomes part of the patient file which is stored as paper for 2 years then copied on to microfilm.

Procedure

The key steps in the procedure are described below. Since this assay is labor intensive and technically difficult, the reader is encouraged to call (651-291-6797) for additional details prior to performing this assay.

1. On the day prior to testing, microtiter immuno-modular strips (Nunc, Denmark) are coated with an anti-mouse Ig polyclonal antibody in a carbonate coating solution.
2. Cells are incubated with human serum for 30 min. at 37° C. Three types of cells are used: 1) homozygous for NA1, 2) homozygous for NA2, and 3) positive for NB1.
3. After washing with 0.2% BSA-saline, a second incubation for 30 min. at 37° C with monoclonal antibody specific for the neutrophil antigen being tested is performed. Various monoclonal antibodies are used in different cell reaction mixtures.
4. The cells are again washed, then solubilized by adding a lysis buffer.
5. After centrifugation of the lysate at 14,000 g, the supernatant is transferred to a separate tube and diluted.
6. After treatment of the immuno-module strips with TRIS buffered saline, the diluted supernatant is transferred to duplicate wells of the immuno-module strips.
7. The immuno-module strips are incubated at 4° C for 90 min.
8. The immuno-module strips are washed with Tris buffered saline.
9. Alkaline phosphatase labeled anti-human Ig is added to all wells and incubated for 90 min at 4° C.
10. After washing the strips with Tris buffered saline, para-nitrophenyl phosphate is added to all wells. This is incubated for 30 min at 37° C.
11. The reaction is stopped with 3M NaOH.
12. The wells are read for optical density at a wavelength of 405 nm with a blank subtracted out.

Interpretation

Results are considered positive if the absorbance is at least 0.250 and three times the negative control.

Troubleshooting

Appropriate dilutions of the various monoclonal antibodies and the alkaline phosphatase labeled anti-human Ig is determined by checkerboard titrations. The dilution should allow maximum O.D. by a positive control with minimum O.D. by the negative control.

Limitation of Procedure

Occasional false negatives may occur if steric inhibition takes place between the human alloantibody and the monoclonal antibody.

References

MONOCLONAL ANTIBODY IMMOBILIZATION OF GRANULOCYTE ANTIGENS (MAIGA)

1. Bux J, Kober V. Kiefel V, and Mueller-Eckhardt C. Analysis of granulocyte-reactive antibodies using an immunoassay based upon monoclonal-antibody-specific immobilization of granulocyte antigens. Transfusion Med, 1993, 3, 157-162.
2. Koene HR, de Haas M, Kleiger M, Roos D, von dem Borne AE. NA-phenotype-dependent differences in neutrophil FcgRIIIb expression cause differences in plasma levels of soluble FcgRIII. British J of Hematol, 1996,93, 235-241.
3. Bux J. Challenges in the determination of clinically significant granulocyte antibodies and antigens. Transfusion Med Rev, 1996, 3, 222-232.

GENERAL

1. Bux J, Stein EL, Bierling P, Fromont P, Clay ME, Stroncek DF, Santoso S: Characterization of a new alloantigen (SH) on the human neutrophil FcgReceptorIIIb. Blood 89:1027-1034, 1997.
2. Huizinga TWJ, Kleijer M, Tetteroo PA, Roos D, Von dem Borne AEGKr: Biallelic neutrophil NA-antigen system is associated with a polymorphism on the phospho-inositol-linked Fc gamma receptor III (CD16). Blood 75:213-217,1990.
3. Koene HR, Kleijer M, Roos D, de Haas M , Von dem Borne AEGKr. FcgRIIIB gene duplication: evidence of presence and expression of three distinct FcgRIIIB genes in NA(1+2) SH(+) individuals. Blood 91:673-679, 1998.

4. Ory PA, Clark MR, Talhouk AS, Goldstein IM: Transfected NA1 and NA2 forms of human neutrophil Fc receptor III exhibit antigenic and structural heterogeneity. Blood 77:2682-2687, 1991.
5. Stroncek DF, Shankar RA, Herr GP. Quinine-dependent antibodies to neutrophils react with a 60 kD glycoprotein on which NB1 antigen is located and an 85 kD glycosyl-phosphatidylinositol linked N-glycosylated plasma membrane glycoprotein. Blood 81:2758-2766, 1993.
6. Von dem Borne AEGKr, de Haas M, Simcek S Porcelijn L, Van der Schoot E. Platelet and neutrophil alloantigens in clinical medicine. Vox Sang 70:34-40, 1996.

SINGLE STEP SEPARATION

1. English D, Anderson BR: Single-step separation of red blood cells, granulocytes, and mononuclear leukocytes on discontinuous density gradients of Ficoll-Hypaque. J Immunol Methods 5:249, 1974.
2. McCullough M, Clay M, Press C, Kline W: *Granulocyte Serology: A Clinical and Laboratory Guide*. American Society of Clinical Pathology, p 157, 1988.

GRANULOCYTE AGGLUTINATION ASSAY

1. Lalezari P: Neutrophil and platelet antibodies in immune neutropenia and thrombocytopenia. in Rose NR, Freidmann H (eds). Manual of Clinical Immunology, Washington DC, American Society for Microbiology, p 630, 1986.
2. McCullough J, Clay M, Press C, Kline W: *Granulocyte Serology: A Clinical and Laboratory Guide*. American Society of Clinical Pathology, p 168, 1988.

INDIRECT GRANULOCYTE IMMUNOFLUORESCENCE ASSAY

1. McCullough J, Clay M, Press C, Kline W: *Granulocyte Serology: A Clinical and Laboratory Guide*. American Society of Clinical Pathology, p 180, 1988.
2. Press C, Kline WE, Clay ME, McCullough J: A microtiter modification of granulocyte immunofluorescence. Vox Sang 49:110, 1985.

Fluorochromatic Microgranulocytotoxicity

Prema R. Madyastha

Purpose

Granulocytes express antigens that are shared with other tissues or cells and also possess antigens that are unique to granulocytes only. In addition to granulocyte-specific antigens that are expressed by all granulocyte series (neutrophils, eosinophils and basophils), neutrophil-specific antigens are detected only on neutrophils.[4,8,9] Under certain pathological conditions or incompatible situations, these antigens stimulate the production of antibodies that cause alloimmune neonatal neutropenias, auto-immune neutropenias in young children,[4,6] autoimmune neutropenias secondary to other diseases or drugs[3] and also febrile or pulmonary transfusion reactions. Thus, it is increasingly recognized in recent years that detection of granulocyte antibodies are crucial in the diagnosis of immune granulocytopenias[8] or transfusion reactions. Clinically significant granulocyte antibodies predominantly are IgG, although IgM and IgA antibodies occur with some frequency, indicating the necessity for inclusion of assays capable of their detection in screening protocols.[5,6] The widely employed techniques are granulocyte agglutination, immunofluorescence, either manual or by flow cytometry, and complement-dependent granulocytotoxicity.[1,47,8,10-12] Granulocyte agglutinins are usually IgG, although IgM and mixtures of IgG, IgM and IgA have been reported.[6] Granulocytotoxins are usually IgM, but may occasionally be IgG.[9] The majority of neutrophil specific antigens were identified by granulocyte agglutination and/or immunofluorescence techniques.[2] Few attempts were made to identify granulocyte specific antigens by granulocyte cytotoxicity technique.[11] The specificity of the antigens or antibodies detected by these techniques may be different, may have different modes of immune destruction or may have different clinical significance. This chapter describes a double fluorochromatic complement dependent microgranulocytotoxicity technique.

Complement dependent microlymphocytotoxicity is the widely used technique to detect HLA antibodies. Based on these principles, granulocytotoxicity assays utilizing complement have been developed and a modified technique was successfully employed by Thompson et al., to detect granulocytotoxins in several patients with granulocytopenia. Granulocyte cytotoxicity assays involve the interaction of cells expressing the target antigens, and serum suspected to contain the antibodies. Thus, the cells are first incubated with the sera to allow the binding of antibodies to the surface antigens. Rabbit complement is then added to the system and incubated further. Since these antibodies are capable of fixing complement, they can thus activate the complement cascade and cause membrane damage. This allows the penetration of suitable vital dyes like trypan blue or eosin which are then added. Live cells exclude the dye and appear colorless whereas the dead cells absorb the dye and appear blue or red. The percentage of live and dead cells are counted using light microscope. By determining the ratio of live and dead cells, the strength of the antibody is measured. A modification of this assay is the fluorochromasia granulocytotoxicity involving double staining. The utilization of diacetyl fluorescein to give green fluorescence to viable cells and ethidium bromide to give red fluorescence to dead cells further enhances the reproducibility of this assay. This technique further employs micro quantities of serum and cell suspension and is less time consuming and thus very suitable for detecting granulocytotoxic antibodies in patients suspected to have immune granulocytopenia.

Specimen

Acceptable Specimen

Isolated serum (3-5 cc) or blood collected in red-top tube that contains no anticoagulant (5-10cc).

Unacceptable Specimen

1. Serum or blood that has been stored at room temp.
2. Serum or blood that has been stored at 4° C more than 2 days
3. Serum or blood that is grossly hemolyzed.
4. Blood collected in tubes with anticoagulant.

Reagents and Supplies

Supplies

1. Tissue typing microplates (72 wells)
2. Disposable plastic test tubes (16 X 100mm and 10 x 75mm)
3. Pasteur pipettes (long and short)
4. Glass beakers [200 ml (2), 2 liter (1)]
5. Hamilton microsyringe (50 µl capacity to dispense 1 µl/click)

6. Hamilton microsyringe (100 µl capacity to dispense 2 µl/click)
7. Hamilton microsyringe (250 µl capacity to dispense 5 µl/click)
8. Hamilton microsyringe adapters to hold the tips
9. Yellow or white disposable tips compatible with these syringes
10. 1ml microcentrifuge tubes
11. Measuring cylinder (100 ml volume)
12. 50 ml conical plastic centrifuge tubes (sterile)

Chemicals

1. Paraffin oil
2. Fluorescein diacetate (FDA) (Eastman Organic Chemicals)
3. Ethidium Bromide
4. Hanks Balanced Salt Solution (10X)
5. Tris
6. Distilled water
7. Dimethyl sulfoxide (DMSO)
8. Sodium Chloride (NaCl)
9. Ethylenediamine tetra acetate-sodium salt (EDTA-Na3)
10. Acid citrate dextrose (ACD) or
11. Citrate phosphate dextrose (CPD)
12. Methyl cellulose-15 (Fisher)
13. Disposable 10 ml centrifuge tubes

Reagents

Name	Chemical Formula/ Lab Label	Accept. Grade	Health / Safety Precaution	Source	How to Prepare	Acceptable Performance	Storage
Methyl cellulose-15 in saline	MC-15	Not applicable	Non-toxic	In-house	See Prep of Reagents	Should form a clear solution.	Refrig (4° C)
Fluorescein diacetate in DMSO (5 mg/ml)	FDA	Not applicable	DMSO is a skin irritant	In-house	See Prep of Reagents	Should dissolve completely	In the dark at room temp/ cover with alum foil
Tris-Buffered Hanks	TBH	Not applicable	Non-toxic	In-house	See Prep of Reagents	Should form clear solution with pH 7.2	Refrig (4° C)
Fluorescein diacetate in DMSO (5 mg/ml)	FDA	Not applicable	DMSO is a skin irritant	In-house	See Prep of Reagents	Should dissolve completely	In the dark at room temp/ cover with al. foil
Ethidium Bromide (1%) in 5% EDTA-Na_3	E-Br	Not applicable	Mutagenic	In-house	See Prep of Reagents	Should form clear solution	In the dark at 4° C / cover with al. foil
2% EDTA in 1.3% NaCl	2% EDTA	Not applicable	Non-toxic	In-house	See Prep of Reagents	Should form clear solution	Refrig (4° C)
Non-toxic Rabbit complement (pooled)	Rab-C′	Not applicable	Non-toxic	Gibco	See Prep of Reagents	Should be non-toxic and Pre-tested for its complement activity	Frozen in small aliquots at-80° C
Negative control serum (AB serum)	Neg	Not applicable	Infectious	In-house	See Prep of Reagents	Should be non-toxic or negative to granulocytes without test serum	Frozen in small aliquots at -80° C
Positive control serum (Antibody positive pooled serum)	Pos	Not applicable	Infectious	In-house	See Prep of Reagents	Should be 100% positive or cytotoxic with test granulocytes in the presence of complement	Frozen in small aliquots at -80° C

Preparation of Reagents

1. Methyl cellulose-15 (2% in saline)
   a. Take a 200 ml capacity glass beaker.
   b. Weigh 2 gm of methylcellulose-15 and place into the beaker.
   c. Weigh 0.85 gm of sodium chloride and place into the same beaker.
   d. Using a measuring cylinder, add 100 ml of sterile distilled water and stir using a magnetic stirrer.
   e. Keep in the refrigerator for a day or two, until the solution is clear.
   f. Distribute in 20 ml quantities into 50 ml conical centrifuge tubes, label and store in the refrigerator.
2. Tris-buffered Hanks (TBH) (100 ml)
   a. Take a 200 ml glass beaker.
   b. Place 10 ml of Hanks balanced salt solution (10X)
   c. Add 5 ml of 2% methylcellulose-15.
   d. Add 1 ml M-Tris base.
   e. Adjust the pH to 7.2 while stirring in a magnetic stirrer.
   f. Pass through a 0.2µm filter, label and store at 4° C in a sterile storage bottle.
3. Fluorescein diacetate (FDA) (0.5%)
   a. Take a 10 ml disposable 16 x 100mm test tube with the cap.
   b. Weigh 25 mg of FDA and place into the test tube.
   c. Add 5 ml of DMSO, place the cap and mix in the vortex mixer.
   d. Label, cover with aluminum foil and store at room temperature in the dark.
4. Ethidium Bromide (EB) (1% in 5% EDTA-Na_3):
   a. First prepare 5% EDTA-Na_3 in 0.145M NaCl
   b. Then dissolve 1% ethidium bromide in 5% EDTA.
   c. Label and freeze in 1 ml aliquots at -80° C and keep 1 aliquot at 4° C in darkness for daily use.
5. EDTA-Na_3 in 1.3% NaCl: (1,000 ml)
   a. Take a 2 liter beaker.
   b. Weigh 20 gm EDTA-Na_3 and place into the beaker.
   c. Weigh 13 gm of NaCl and place into the same beaker.
   d. Add 1 liter of distilled water and stir in the magnetic stirrer until clear solution is obtained.
   e. Slight warming may be needed to completely dissolve the EDTA.
   f. Label and store at room temperature.
6. Rabbit complement:
   a. Rabbit complement should be always pretested routinely and should be ascertained that it is not toxic by itself to the normal granulocytes in the absence of positive serum. Complement should also be evaluated for its appropriate dilution in which maximum cytotoxicity is obtained with the positive cytotoxic serum without the interference of prozone phenomenon. If granulocytotoxicity is observed, toxic factors can be removed by absorption with pooled human red cells as detailed below:
   b. Prepare washed, packed human red blood cells.
   c. Mix equal volume of packed red cells and the rabbit serum to be absorbed.
   d. Incubate for 45 min at 0° C.
   e. Centrifuge at 1,000 x g at 4° C for 5min to remove the red cells. The absorbed complement should be retested for its non-toxic nature. If found toxic, a second absorption may be performed as above.
   f. Once the complement is free from toxicity and found to contain complement activity, store in small aliquots at -80° C.
7. Negative and Positive control sera:
   a. Collect 10-20cc blood in plain tubes from a healthy, non-transfused, normal donor of AB blood group. Separate the serum, inactivate and test in undiluted at a 1:4 dilution for inherent toxicity using normal granulocytes and rabbit complement. If it is found to be negative, aliquot in small quantities and freeze at -80° C. Use either undiluted or 1:4 as a negative control serum.
   b. Sera from recipients of multiple whole blood or granulocyte transfusions, from kidney and bone marrow allograft recipients, and from immunoneutropenic patients are initially screened to identify prospective granulocytotoxic antibody. Choose strongly reactive lymphocytotoxic sera and retest with additional unrelated cell donors to identify provisional clusters. Pool several positive sera to obtain multispecific and multireacting serum and determine the appropriate dilution. Use this as a positive control in the assay.

Instrumentation/Special Equipment

1. Vortex mixer
2. Table top centrifuge with rotor capable of holding appropriate tubes and trays.
3. Fluorescence microscope (inverted preferred), equipped with excitation
4. and barrier filters to permit simultaneous visualization of green and red fluorescence
5. -80° C freezer, Refrigerator
6. 0.2 µm micropore filter
7. Magnetic stirrer

Calibration

1. Table top centrifuge should be calibrated for its accuracy of speed once in six months. This can be done by biomedical personnel, or it can be contracted out. A signed report should be maintained in the lab.
2. The temperature of the freezer and refrigerator should be checked once a month and a log of the performance should be maintained in the lab.
3. A log should be maintained for the hours used for the fluorescence microscope.
4. The microscope also should be calibrated by the a representative from the company where the scope was purchased.

Quality Control

1. The pH of the TBH (7.2) should be checked once in 2 weeks and a log maintained. It should be also checked for any fungal or bacterial growth. When it does not meet the requirements fresh reagent should be prepared.
2. There is no need to do any additional QC for the other reagents. As soon as the reagents are freshly prepared, its performance should be checked using a normal donor and entered in the log book. After this initial recording, a log should be maintained once a month for the performance of the reagents whenever a patient specimen is tested. In suspected circumstances, a special QC may be performed and if needed fresh reagents may be prepared.
3. Rabbit complements, AB serum and the positive serum should be tested as soon as they are procured, against at least with 3-5 normal donor granulocytes. Rabbit complement should be non-toxic and should give negative values with normal donor granulocytes in the absence of cytototoxic serum. AB serum also should give negative values with normal donor cells in the presence of complement. Positive serum should give strong positive cytotoxicity in the presence of complement. Once found suitable, the dilution giving the optimum reactivity should be maintained in the log book. Thereafter, their performance while testing the patient sera should be separately recorded in the QC log book once in two weeks and monitored. Once in six months or whenever there is a problem, they should be tested against normal donors. If found unsuitable, fresh reagents should be procured.

Procedure

1. Tray preparation
   a. Using Hamilton syringe (100 µl) and a disposable tip, place 4 µl of paraffin oil to each well of the microplate.
   b. Using Hamilton syringe (50 µl) and a disposable tip, place 1 µl of the test sera, undiluted or diluted under the oil in the middle of each well. Change the tip each time before transferring another either the test serum, or the control sera. Place 1 µl of the appropriate predetermined dilution of negative and positive control sera in suitable wells.
   c. Store the tray at 4° C for use the same day, or at -80° C for longer periods.
2. Granulocyte preparation
   a. Obtain fresh blood anticoagulated with ACD or CPD.
   b. Separate the granulocytes using the Ficoll-Hypaque double density gradient centrifugation described in the earlier section.
   c. Wash the cells using 10 ml TBH twice, centrifuging each time at 1500 rpm for 5 min at room temp.
   d. Resuspend the cells in 1 ml TBH and determine the cell concentration using hemocytometer.
   e. Adjust the concentration between 3-5 x 10^6/ml using TBH.
3. Cytotoxicity assay
   a. Thaw the antisera tray 10-15 min before use.
   b. Label the cells by adding 2 µl of FDA to each ml of cells.
   c. After 1-2 min, centrifuge the cells at 1000 rpm for 1 min and remove the excess FDA supernatant.
   d. Resuspend the cells in TBH to the original volume and concentration.
   e. Using a Hamilton syringe (50 µl), place 1 µl of the FDA-labeled cells to each well containing the test sera.
   f. Mix the sera and cells by gentle circular rotation of the tray on the bench.
   g. Incubate the trays at room temp in the dark for 30 min.
   h. Wash each well twice with TBH by adding one drop using a long Pasteur pipet. Using a fresh Pasteur pipet, aspirate the TBH from each well by carefully touching the top of the well with the tip of the Pasteur pipet.
   i. Thaw rabbit complement, and with the Hamilton microsyringe (250 µl capacity), add 5 µl of the properly diluted complement to each well.
   j. Incubate for 60 min at room temp in the dark.
   k. Wash the plates 2-3 times with 2% EDTA in 1.3% NaCl by flooding the plates gently using a 50 ml beaker.
   l. Wash until all the oil is removed. Remove the excess medium using a Pasteur pipet as before.
   m. Add 2 µl of freshly thawed 1% EB in 5% EDTA and allow 10 min for staining of dead cells to occur.
   n. Wash two times with 2% EDTA in 1.3% NaCl as in step j.
   o. Centrifuge the tray for 5 min at 400 x g to settle the cells before reading.
   p. Flick the plate gently to remove the supernatant medium.
   q. Estimate the degree of viability by counting the number of viable and dead cells using the fluorescent microscope.

Calculations

Read the reactions within 2 hours. Viable granulocytes appear as bright green fluorescing cells. The non-viable cells take up the EB and appear red.

1. View a total of at least 100-200 cells and count the number of dead cells. Score the reactions according to the percentage of dead cells.
2. First score the negative control and then positive control. These two controls should give the expected values. Then proceed to scoring the test sera.
3. Give the percentage of dead cells in each of the wells and estimate their gradings.

Results and Interpretation

The reactions are scored and graded as follows (by estimation of differential red-green dye uptake):

% Dead	Score	Interpretation
<10%	1	Negative
11-20%	2	Negative
21-40%	4	Weakly Positive
41-80%	6	Positive
>80%	8	Strongly positive

Procedure Notes

1. Since EDTA is anticomplementary, and microgranulocytotoxicity involves the use of complement, for granulocyte isolations, blood specimens should be collected in tubes containing either ACD or CPD as an anticoagulant without EDTA.
2. If no granulocyte specific sera are available, a high titered polyspecific anti-HLA antiserum that previously has been shown to contain anti-granulocyte activity may be used as a positive control.
3. Negative and positive sera and rabbit complement are always pretested and should be used in the predetermined dilution to give optimum reactivity. TBH should be used to dilute these reagents.
4. The negative control serum should always give less than 20% dead cells. In a well controlled setting, the author always obtained less than 5% dead cells in negative control serum. Positive serum should always give more than 80% dead cells.
5. Bovine Serum Albumin (BSA)-Phosphate buffered saline (PBS) may be substituted for Tris-Buffered HBSS.
6. In place of FDA, carboxyfluorescein diacetate may be used where available. It is considerably more stable and permits a greater latitude in time between staining and scoring.
7. Both fluorescein dyes may be dissolved in acetone and stored in the dark at -20° C or in DMSO and stored in the dark at room temperature.

Limitations of Procedure

1. Complement is the most critical variable for the successful performance of the granulocytotoxicity test and several precautions must be taken to assure acceptable reproducibility. First, the native rabbit serum must be titered in HBSS (other calcium containing diluents may be substituted) and tested with both strong and moderately weak alloantisera against known positive and negative cell donors. Many lots are innately toxic and must be discarded. The use of rabbit anti-human granulocyte serum as a positive control for complement titration is misleading and may lead to an inappropriate selection. Absorption of the complement with fresh autologous human red blood cells may remove nonspecific toxicity, but many pools of sera from young rabbits are quite satisfactory without absorption. Preliminary testing to discard toxic lots and selecting complement on the basis of titration against alloantisera is more efficient.
2. A second very important variable involves the washing step following antisera incubation and prior to complement addition. Many anti-complementary sera completely prevent complement lysis in the absence of the washing step. These same precautions have proven to be equally critical for the successful performance of the cytotoxicity test.
3. Heparinized blood yields granulocytes that may give highly variable cytotoxic responses to some antisera. Acid citrate dextrose (ACD) or citrate phosphate dextrose gives a better performance with high yield of cells. Since EDTA is anticomplementary, it should be avoided when cells are used for complement dependent cytotoxicity assays. Some, however, believe that EDTA can be removed from the cell surface by washing.
4. The use of fresh blood is absolutely essential since the half life of granulocytes is only 6 hrs even under physiologic conditions.
5. HLA class I antigens are present on granulocytes but in very low concentration. Consequently many anti-HLA reagents are not cytotoxic for granulocytes. Positive sera cannot be presumed to be granulocyte specific, unless granulocytes and lymphocytes from the same individuals are run in parallel or the sera are absorbed with lym-

phocytes. Additionally, cytotoxicity generally does not correlate with agglutination, i.e., they detect different antigen systems.

References

1. Blaschke J, Severson CD, Goeken NE and Thompson JS, Microgranulocytotoxicity. J Lab Clin Med 90:249, 1977.
2. Bux J and Chapman J Report on the second international granulocyte serology workshop. Transfusion 37:977-983, 1997.
3. Guffy MM, Goeken NE and Burns CP, Granulocytotoxic antibodies in a patient with Propylthiouracil-induced agranulocytosis. Archives of Internal Medicine 144:1687, 1984.
4. Madyastha PR, Kyong CU, Darby CP et al., Role of neutrophil antigen NA1 in an infant with autoimmune neutropenia. Am J Dis Child 136:718-721,1982.
5. Madyastha PR, Madyastha KR, Wade T and Levine DH, An improved method for rapid layering of Ficoll-Hypaque double density gradients suitable for granulocyte separation. J Immunol Meth 48:281-286, 1982.
6. Madyastha PR, Fudenberg HH, Glassman AB, Madyastha KR and Smith CL, Autoimmune neutropenia in early infancy: a review. Ann Lab Clin Sci 12:356- 367, 1982.
7. Madyastha PR and Glassman AB, Detection of granulocyte antibodies by flow cytometry. Ann Lab Clin Sci 17:267-268, 1987.
8. Madyastha PR and Glassman AB, Neutrophil antigens and antibodies in the diagnosis of immune neutropenias. Ann Clin Lab Sci 19:146-154, 1989.
9. McCullough J, Clay M, Press C and Kline W, Granulocyte Serology: A Clinical and Laboratory Guide. Amer Soc Clin Pathol, pp 176-179, 1988.
10. Thompson JS, Herbick JM, Burns CP, Strauss RG, Blaschke JW, Koepke JA, Maguire LC and Goedken MM, Granulocyte specific antigens detected by microgranulocytotoxicity. Transpl Proc 11:431, 1979.
11. Thompson JS, Oerlin VL, Herbick JM, Severson CD, Claas FHJ, Amaro JD, Burns CP, Strauss RG and Koepke JA, New granulocyte antigens demonstrated by a microgranulocytotoxicity assay. J of Clin Invest 65:1431, 1980.
12. Thompson JS, Overlin V and Severson CD et al., Demonstration of granulocyte, monocyte and endothelial cell antigens by double fluorochromatic microcytotoxicity testing. Transpl Proc 12:26-31, 1980.

Monocyte Cytotoxicity

Peter Stastny

Purpose

Preparations of monocytes can be used as targets for cytolytic T cells or of cytotoxic antibodies. The complement-mediated cytotoxicity procedure to be described here is simply a modification of the standard microcytotoxicity method originally developed with lymphocytes.

Specimen

Peripheral blood

Unacceptable Specimen

Sample more than 24 hrs old

Instrumentation

1. Tubes, 15 ml conical
2. Microliter syringes
3. Microtest trays
4. Phase-contrast microscope

Reagents

1. RPMI 1640 medium with 10% normal human serum
2. Rabbit complement, titrated for monocyte cytotoxicity
3. 5% Eosin solution
4. Formaldehyde, pH 7.2-7.4

Procedure

1. Suspend monocytes at a concentration of 3.0 x 10^6/ml in RPMI 1640 containing 10% normal human serum.
2. Add 1 ml of monocyte suspension to preloaded trays containing 1 ml serum in each well.
3. Incubate for 1 hr at room temperature (RT).
4. Add 5 ml of selected rabbit complement and incubate for an additional 2 hrs at RT.
5. Stain with eosin and fix with formaldehyde as in the standard microcytotoxicity method.
6. Allow cells to settle and then read results using an inverted phase-contrast microscope.
7. Score cytotoxicity on the basis of percent cells killed.

Controls

Negative Control

Consists of normal human serum. It is used to determine viability of the monocyte preparation at the end of the cytotoxicity procedure.

Positive Control

A broadly-reactive anti-class I HLA serum and a monomorphic antibody that kills monocytes but does not react with other cells.

HLA Class II Control

Sometimes it is useful to include antibodies to HLA-DR (for example, L243) which react with all monocytes and to HLA-DQ (for example, TU22) which do not react with the majority of unstimulated human monocytes.

Other controls are included depending on for what the monocyte cytotoxicity test is being used.

Troubleshooting

1. Low viability – Most often this is due to problems in the course of isolation of the cells. However, it should be remembered that some batches of rabbit complement, which are adequate for working with lymphocytes, may be toxic for monocytes.
2. Reactions are weak – Some batches of rabbit complement do not work properly in cytotoxicity tests using monocytes as targets. Complement activity can be checked in parallel testing on DR trays using B cells and monocytes from the same donors. Such tests should give high correlation between B cell and monocyte positive reactions.

Interpretation

Clear cut positive and negative reactions should be obtained. In order to determine whether a cytotoxic antibody is monocyte specific, it is necessary to know whether it reacts with T cells and B cells from the same donor. It is customary to absorb sera with platelets to remove class I HLA antibodies and with B cells to eliminate anti-DR. Nevertheless, it is advisable to test monocytes, T and B cells in parallel from the same donor to evaluate the significance of the monocyte reactions.

Common Variations

Other methods of cytotoxicity may be used including fluorochromasia with separated monocytes and the two-color cytotoxicity procedure with unseparated preparations of mononuclear cells from peripheral blood.

References

1. Colbaugh P, Stastny P: Antigens in human monocytes, III. Use of monocytes in typing for HLA-D related (DR) antigens. Transpl Proc 10:871, 1978.
2. Moraes JR, Stastny P: A new antigen system expressed in human endothelial cells. J Clin Invest 60:449, 1977.

The Use of Cultured Fetal Cells, Non-Lymphoid Tumor Cells and Fibroblasts for HLA Typing

Marilyn S. Pollack

This section will briefly describe procedures that can be used to establish and maintain cultured human fetal cells, human tumor cell lines or fibroblast lines; to prepare appropriate cell suspensions for HLA typing; and, the HLA typing methods that can be used. This section is based on three different articles from the 3rd Edition of the ASHI Laboratory Manual, as noted in the Acknowledgment section below.

Purpose

Fetal Cells

HLA typing of cultured fetal cells has been used for several clinical purposes. Such typing has been used to establish whether or not the fetus is affected in a pregnancy at risk, because of the previous birth of an affected child, for closely HLA-linked monogenetic recessive diseases. Those most often involved are: congenital adrenal hyperplasia due to 21-hydroxylase (21-OH) deficiency[1-3] and complement C4 deficiency.[4] Although direct molecular methods are now available, the prenatal diagnosis of 21-OH deficiency often continues to require HLA typing because mutations in the 21-OH gene cannot always be detected by molecular techniques. HLA typing is no longer used to predict inheritance of the HLA-lined disease Spinocerebellar Ataxia since there are much more closely linked markers.[5] However HLA typing of cultured fetal cells is still used for the prenatal determination of paternity, to avoid abortion, for example, in a case involving the rape of a married woman,[6] and recently has become an established procedure for prenatal determination of fetal HLA identity to a sibling for whom a neonatal cord blood stem cell transplant would be an important therapeutic option.[7,8]

Tumor Cells

HLA typing of cultured human tumor cells is used for investigations of cancer patient immunity. In testing for patient antibody or cell mediated immune responses to “tumor antigens” with cultured tumor cell-line target cells, for example, investigators should ascertain the HLA specificity of the allogeneic tumor cell lines they use in order to sort out tumor-specific from allogeneic immune responses.[9-11] When donor lymphocytes have been available for comparative tests, HLA phenotypes have generally been the same on the tumor cells although loss of one or more antigens is occasionally observed.[12-14]

More recently, HLA typing of tumor cell lines has been of interest because it became clear that T lymphocytes recognize antigens when associated with and presented by common HLA molecules (on autologous or allogeneic targets). The antigens recognized are short peptides 9-11 amino acids in length when recognized by cytotoxic T lymphocytes (CTL) in association with Class I molecules HLA-A/B/C, or 14-18 amino acids in length when presented by MHC Class II molecules.[15-16] Since these peptides include the elusive “tumor antigens,” their isolation and characterization depend largely on our ability to determine the HLA-type of human tumors and to use in vitro culture to yield large numbers of tumor cells either from autologous or from allogeneic, HLA-compatible tumors of the same histology. Isolation of HLA molecules of particular interest, using HLA-specific monoclonal antibodies (MoAb), and their tightly bound tumor peptides can then be possible.[17] Our own studies have characterized proliferative responses to the HLA molecules[18-19] and to their HLA-bound peptides using cytotoxic T-cell lines.[20-22]

It has also been shown that patients treated with tumor vaccines show better clinical responses when immunized with HLA-matched allogeneic tumor cells than with cells from randomly chosen tumors.[23-24] We have also made use of the enormous polymorphism of the HLA system to detect a few cases of contaminating tumor cell-line overgrowth or to confirm the donor identity for tumor cell lines.[25]

Fibroblasts

HLA typing of cultured fibroblasts has been extensively used by ourselves and others for studies of the actual function of the HLA molecules themselves. These studies have demonstrated, for example, that there is differential up-regulation of different HLA molecules (DQ less than DR, for example) by activating agents like gamma interferon, and that the

functional expression of HLA molecules may be different on different cell types.[26-27] The HLA typing is necessary in order to demonstrate the functional activity of the HLA molecules either as targets for antibody responses or as activators of cellular responses.

Specimens

Fetal Cells

Clinical considerations involved in the choice between chorionic villus sampling and amniocentesis procedures to obtain fetal cells for prenatal tests are reviewed by Meade, et al.[28]

Amniotic Fluid Cells

Although both epitheloid and fibroblastic fetal cells are present in amniotic fluid samples, usually only fibroblastic cells adhere to the flasks and grow well using the culture techniques described below. Amniotic fluid for the establishment of cultured amniotic fluid cells for prenatal HLA typing is generally obtained between the 14th and 20th weeks of gestation, by standard amniocentesis techniques. At least 20 ml of fluid should be obtained, if possible, and if more fluid is available, the time needed to grow sufficient cells for testing is correspondingly reduced.

Chorionic villus samples

These can be obtained during the 9th-11th weeks of gestation. Since pure cultures are very difficult to establish because of the need to carefully separate the maternal and fetal cell layers, THESE CULTURES SHOULD ONLY BE ESTABLISHED BY AN EXPERIENCED TECHNOLOGIST IN A CYTOGENETICS LABORATORY (procedure not provided here).

Fetal Fibroblasts

Fetal fibroblast cultures can be established from virtually any portion of the fetal tissue that can be obtained following an abortion. Liver tissue should be avoided.

Tumor Cells

These are generally obtained as surgical specimens or collected from ascites fluid or pleural fluid.

Fibroblasts

These are generally obtained as surgical specimens or as skin punch biopsies from volunteers.

Reagents and Supplies

Establishment of Cultures

1. Fetal Bovine Serum (FBS)
   FBS should be heat-inactivated at 56° C for 30 min, filtered through, 0.8, 0.45 and finally a 0.22µ Millipore filter and aged at 4° C for two weeks to eliminate mycoplasma. Each batch of FBS should be prescreened for its ability to support growth of established human cell lines.
2. Culture Medium for Amniotic Fluid and Chorionic Villus (CVS) Cells
   Complete Chang medium (Hanna Media, Berkeley, CA) mixed with penicillin (100 IU/ml), streptomycin (20 µg/ml) and gentamycin (0.5%) and refiltered (0.22µ filter) before use.
3. Primary Medium
   Using minimum essential medium (MEM) containing 1% non-essential amino acids or RPMI 1640, add L-glutamine (2 mM), penicillin (100 IU/ml), streptomycin (100 mg/ml), gentamycin (0.5%) and FBS (20%) and refilter (0.22µ) before use.

   NOTE: In addition to MEM, in certain cases, tumor cell growth will benefit from culture in richer media such as Dulbecco's MEM (D-MEM) or RPMI 1640.[29] D-MEM with its high glucose content may be suitable for fast-growing tumor cells. Furthermore, if a CO_2 incubator is not available, or if, in certain instances, there are concerns about the sterility or possible contamination of the cultures in a CO_2 incubator, L-15 medium offers a good alternative to MEM/RPMI 1640.
4. Maintenance Medium
   Primary Medium containing 10% FBS.
5. Hanks' Balanced Salt solution (HBSS) – Standard Cell Culture medium.
6. Ethylenediaminetetraacetic acid (EDTA)
   2 g EDTA in 1000 ml of Hanks BSS. Aliquot in 100 ml bottles. Store at -20° C.
7. Trypsin – EDTA
   Prepare a 1X (0.25%) stock solution by mixing 100 ml 10X trypsin (2.5%)(Gibco) with 100 ml EDTA and 800 ml HBSS. Aliquot in 100 ml bottles and store -20° C. Filter before using. After thawing, store at 4° C.

8. Barbitol Buffer
   Dissolve one tablet (Oxoid, Limited) in 100 ml of warm distilled water. Adjust pH to 7.2 with 1N sodium hydroxide (NaOH) or 1M hydrochloric acid (HCl) as appropriate. Filter and store (indefinitely) at room temperature.
9. Gamma Interferon (IFN-γ)
   Follow manufacturer's instructions for dilution and storage, e.g., dilute to 100,000 U/ml in 1 ml vials and store at 4° C. (Do not refreeze after thawing.)
10. McCoy's 5a Medium
11. Phosphate buffered saline (PBS)
    1.70 g of Sodium Chloride (NaCl), 12.8 ml 1M sodium phosphate (NaH_2PO_4) (1.3 g/50 ml Distilled water), 67.2 ml. 1M Na2HPO4, dilute to 8 liters with distilled water, adjust pH to 7.4 with 1N NaOH (10 g/250 ml distilled water) or 1M HCl, as appropriate.
12. Collagenase
13. Deoxyribonuclease enzyme (DNAse) type I
14. Histopaque
15. Fluorescein diacetate solution (for serologic typing)
    Prepare a stock solution by dissolving 10 mg of fluorescein diacetate in 2 ml of acetone. Cover with foil and store at -20°c.
16. Ethidium bromide (EB) solution (for serological typing)
    Prepare a 0.025% solution of EB in RPMI 1640 (e.g., 50 mg in 200 ml). Cover with foil and store at 4°c. (Note: Use a face mask when weighing the power.)
17. Specific HLA Class I typing sera or standard (commercially available) Class I typing trays and appropriate complement if using serological test procedures
18. Appropriate primer pairs, probes and other necessary reagents if using molecular typing methods

Instrumentation

1. Sterile hood
2. Humidified CO_2 incubator
3. Centrifuges
4. Inverted phase contrast microscope and other equipment for serological HLA typing
5. Equipment for molecular HLA typing

Procedures

Note: All Procedures involving establishment and culture of cells must utilize sterile techniques.

A. Establishment of Amniotic Fluid Cell Cultures

1. Centrifuge the fluid for 10 min. at 1000 rpm to pellet the cells; save approximately 2 ml of fluid per 10 ml original volume.
2. Dilute the fluid with an equal volume of complete Chang medium (Chang A + Chang B, Hanna Media), which already contains 8% serum, adding 100 IU/ml penicillin, 10 mg/ml streptomycin, 0.5% gentamycin solution, and 10% additional fetal Bovine Serum (FBS), and resuspend the pelleted cells.
3. Disperse the fluid into 3 to 4 different T-25 flasks under sterile conditions, approximately 4 ml per flask. Different flasks are used to assure that at least one flask will remain sterile.
4. Incubate the flasks under sterile conditions at 37° C in a humidified CO_2 incubator until most of the viable amniotic fluid cells become attached to the bottom of the flask. This usually takes 3-7 days. After 5 days, add 2 ml fresh Chang medium with antibiotics and 10% FBS; repeat every 2 days until the cells become attached.
5. When the cells become attached, pour off half the diluted amniotic fluid and replace with fresh complete Chang medium with antibiotics. Repeat this procedure every 4 days.
6. When the monolayers approach confluence, pour off the medium and trypsinize the cultures with warm (37° C) trypsin-EDTA (5 ml) – just long enough to allow most of the cells to detach from the flask (approximately 2 min. at 37° C).
7. Immediately add 5 ml McCoys 5a medium or similar medium containing 20%-30% fetal calf serum (FCS) to prevent further activity of the enzyme and wash the cells once with the same medium, pelleting the cells at 800 rpm for 5 min., before reculturing in additional new flasks with complete Chang medium.
8. When confluent, the cultures should be split into two subcultures each, with addition of 1000 U/ml IFN-g to some flasks 24-48 hrs after subculturing when there are sufficient cells for typing, if typing will be done using serological methods. (see Typing procedures).

B. Maintenance of Cultured Amniotic Fluid Cells and CVS Cells (cultures established elsewhere)

1. To "feed" the cells between passages, replace all the Chang medium with fresh medium every 2-3 days until the monolayers again approach confluence. At that time, trypsinization and subculture should be repeated.
2. After approximately 4-8 passages (4-8 weeks of culture) the cells become vacuolated and cease to grow. HLA typing tests should have been completed if possible, before this stage is reached.

C. Establishment of Tumor Cell Lines from Surgical Specimens

1. Wash surgical specimens in phosphate buffered saline (PBS) containing penicillin (100 IU/ml) and streptomycin (100 µg/ml), and dissect free of fat, normal tissue and necrotic tissue.
2. Repeat the wash step and finely mince the tumor fragments.
3. If very few cells are released, treat the fragments with 1X (0.25%) trypsin and 0.02% collagenase for 15-30 min. at 37° C with continuous mixing using a magnetic stirrer with slow motion. Digestion with enzymes can be prolonged for up to 4 hrs, if more tumor cells are needed. However, cell destruction under these circumstances releases high molecular weight DNA. To avoid additional loses due to the attachment and clumping of cells to the DNA, good results are obtained if tissue digestion is performed in the presence of 0.002% deoxyribonuclease type I (100 U/mg) in addition to trypsin and collagenase. Add RPMI 1640, PBS, or McCoys medium with 20%-30% FBS to stop the trypsin action.
4. Wash the cell suspensions and small fragments 2-3 times and suspend in MEM containing 1% non-essential amino acids, 2mM glutamine, 100 IU/ml penicillin, 100 mg/ml streptomycin, 0.5% gentamicin and 20% FBS (Primary Medium).
5. Allow fragments to settle for 2 min.
6. Inoculate some T-25 flasks with 1 x 10^6 viable cells from the supernatant and others with 0.2-0.5 ml of the precipitate containing tissue fragments (and some cells).
7. Incubate the flasks at 37° C in a humidified CO_2 incubator.
8. Feed the flasks with fresh Primary Medium twice a week by removing a portion of the spent medium and adding fresh medium. In addition, tumor cells from hormone-dependent tumors which are characterized by slow growth will benefit from supplementing the media with transferrin (0.1 mg/ml), hydrocortisone (0.5 µg/ml), estrogen growth factor (EGF)(5 ng/ml) and insulin (5 µg/ml.)[30]
9. When newly cultured cells become more than half confluent, carefully detach them with 0.10% trypsin at 37° C (leaving the more firmly adherent, contaminating fibroblasts behind) and transfer the detached cells into new flasks.

   Begin initial subculturing 2-12 weeks after seeding the original specimen. After the tenth subculture, feed the cells twice a week with MEM supplemented with 1% non-essential amino acids, 2mM glutamine, 100 U/ml, 100 µg/ml streptomycin, 0.5% gentamicin and 10% FBS (Maintenance Medium). The frequency of subculturing different cultures ranges from once every week to once every 6 weeks depending on the rate of growth. Cultures should be repeatedly tested for mycoplasma, fungi and bacteria. Treat immediately or discard contaminated cultures.

   NOTE: The primary culture of tumor cells can also be accomplished using alternative methods such as cell adhesive matrix growth[31] that improve the yields over the culture of tumor cells on plastic. Another method that has been used is culturing in agar (anchorage independent cell growth)[30] as follows:

   Anchorage – Independent Cell Growth
   a. Prepare a bottom layer of 0.6% agar (Bacto-Agar, Difco) with complete culture medium.
   b. Pour the agar into multi-well tissue culture plates. According to the purpose of the procedure, either 96, or 48, or 24-well plates may be used.
   c. Allow the agar to solidify for 24 hrs at 37° C in an incubator of desired/available type (±CO_2).
   d. Prepare the second layer using a high-quality, low-melting point agar/agarose. The molten agar solution should be in liquid form at 36-38°.
   e. Dilute the viable tumor cell suspension (10-50,000 cells/ml) in pre-warmed (at 37° C) complete cultured medium. Add agar (0.6%) to each cell suspension in equal volume to a final 0.3% concentration. Pour the mixture immediately into the wells that have been pre-coated with 0.5% agar in complete medium.
   f. Incubate the plates at 37° C in 5% CO_2 and observe formation of colonies from clones after 2-4 weeks under the light microscope.

D. Establishment of Tumor Lines from Ascites Fluid or Pleural Fluid Specimens

1. Collect heparinized ascites/pleural fluid (and additional sterile ascites fluid).

   NOTE: When tumor cells are isolated from ascites, usually the ascites fluid contains a mixture of tumor growth factors (e.g., TGF-alpha). Therefore, supplementation of the primary medium with up to 30% autologous sterile-filtered ascitic fluid will help the growth and the establishment of primary tumor lines.
2. Dilute heparinized fluid 1:1 with sterile PBS.
3. Centrifuge 10 min. at 800 x g (approximately 2000 rpm).

   NOTE: The cell pellet usually contains tumor cells with contaminating lymphocytes, erythrocytes, and granulocytes. Since culture of tumor cells together with immune cells may ultimately lead to destruction of tumor cells, two additional steps must be taken.
4. Suspend the pellet in 5-10 ml culture medium or PBS without FCS, and overlay on 100% Ficoll-Hypaque (density gradient 1.077).
5. Centrifuge for 30-40 min. at 400 x g (approximately 1500-1600 rpm).
6. Discard the erythrocytes, granulocytes, debris, and large tumor clumps that sediment to the bottom of the tube.

7. Collect the isolated tumor cells and lymphocytes that remain at the interface medium – 100% Histopaque. If fewer than 50% of the cells are lymphocytes, culture cells as per steps 6-10 in section E above, using medium supplemented with ascites fluid, if appropriate. If more than 50% lymphocytes are collected with the tumor cells, then first proceed as follows below:
8. Prepare a two-step gradient by overlaying 75% Histopaque (7.5 ml Histopaque + 2.5 ml PBS) over 100% Histopaque.
9. Collect the cells at the interface medium – 100% Histopaque and gently add on top of the 75% Histopaque.
10. Centrifuge the tubes at 800 x g for 20 min. and collect the interface medium – 75% Histopaque which is enriched in tumor cells (the interface 75% – 100% Histopaque is enriched for lymphocytes).

NOTE: This separation step can also be used for separation of leukocytes from tumor cells isolated from solid tumors. Characterization of growing cells as of tumor origin needs, as an ultimate test, karyotype analysis. Alternatively, monoclonal antibodies that preferentially stain tumor cells, compared with fibroblasts or mesothelial cells can be used.

E. Establishment of Fibroblast Cell Lines

1. Wash tissue specimens in PBS containing penicillin (100 IU/ml) and streptomycin (100 µg/ml)
2. Repeat the wash step and finely mince the tissue fragments.
3. Wash the cell suspensions and small fragments 2-3 times and suspend in Primary Medium.
4. Allow to settle for 2 min.
5. Inoculate some T-25 flasks with 1 x 10^6 viable cells from the supernatant and others with 0.2-0.5 ml of precipitate containing tissue fragments and cells.
6. Incubate the flasks at 37° C in a humidified CO_2 incubator.

F. Maintenance of Fibroblast Cell Lines

1. Feed the flasks with fresh Primary Medium twice a week by removing a portion of the spent medium and adding fresh medium.
2. When newly cultured cells become more than half confluent, carefully detach them with trypsin-EDTA at 37° C and transfer the detached cells into new flasks.
3. Begin initial subculturing 2-12 weeks after seeding the original specimen. After the tenth subculture, feed the cells twice a week with Maintenance Medium. The frequency of subculturing different cultures ranges from once every week to once every 3 weeks depending on the rate of growth. Cultures should be repeatedly tested for mycoplasma, fungi and bacteria. Treat immediately or discard contaminated cultures.

G. Preparation of Cell Suspensions for HLA Typing

1. For class I serological typing, pre-culture cells for 2 or 5 days with 1000-5000 U/ml IFN-γ to increase expression of class I HLA antigens. IFN-γ increases the expression of all Class I HLA antigens,[32-34] eliminating previously described[35-36] technical problems relating to poor expression of some HLA-B and all HLA-C Locus antigens (Class I or Class II typing with DNA typing techniques does not require any preincubation step.)
2. Pour off growth medium and trypsinize flask (T-25 flask) during growth phase with 5 ml warm 0.1% trypsin EDTA (1X trypsin diluted 1:25 with HBSS) using the minimum time required to detach most of the cells (approximately 2 min. at 37° C for fetal cells or 5-10 minutes for tumor cells).
3. Immediately add RPMI 1640, McCoy's 5a or other medium with 20%-30% FBS to stop the trypsin action against the cells.
4. For serological class I typing, wash the cell suspension twice with RPMI 1640, McCoy's or other medium containing 20%-30% FBS, centrifuging the cells at low speed between washes (800-1000 rpm for 5 min.). Resuspend the cells in RPMI 1640, McCoy's or other medium with 20%-30% FBS and adjust the concentration to approximately 1 x 10^6 viable cells per ml by counting in a hemacytometer (with trypan blue staining). Allow the cells to "recover" HLA antigens by leaving the suspension at room temperature for 90 min. and then proceed to add the cells to standard preplated HLA class I typing trays. For typing for selected antigens by the two color fluorescence procedure, plate cells sterilely in complete Chang medium into individual wells of a microtiter tray (see procedure).
5. For class I or class II molecular typing, wash the trypsinized cells with any tissue culture medium and use with any standard DNA extraction procedure (see relevant chapters).

H. Serological 2-Color Fluorescence Microcytotoxicity Test Procedure For Cultured Cells with Known Alternative HLA Class I Antigens (e.g., prenatal diagnosis of 21-OH-deficiency):

1. Preplate cells by adding 1 µl (1000 cells) freshly trypsinized amniotic or chorionic villus cells in complete Chang medium or fibroblasts or tumor cells in Maintenance Medium with 10% fetal calf serum to a sterile microtiter plate with 1000-5000 units of IFN-γ per ml.
2. Incubate the tray for 48-72 hrs at 37° C in a humidified CO_2 incubator.
3. Flick off the medium and rinse the cells twice with HBSS by flooding the wells and flicking off the solution.
4. Add 5 µl of selected typing serum to each well and incubate 45 min. at RT.
5. Add 5 µl of pretested complement to each well and incubate 45 min. at RT.

NOTE: Absorbed rabbit complement [rabbit complement absorbed in the presence of EDTA (1/10 volume 0.1M EDTA) at 0° C with approximately ¼ volume pooled leukocytes or relevant test cells] can be used when the cells of a particular tumor cell line are too sensitive for locally or commercially available complement (positive reactions in negative control test wells under all condition). Restore divalent cations by addition of 1/10 volume 1M calcium chloride ($CaCl_2$).

6. Add 1 µl of fluorescein diacetate/acetone stock solution to 2.5 ml EB solution (0.025% in RPMI 1640) diluted with 2.5 ml Barbitol Buffer.
7. Add 10 µl of the dye mixture per well. Incubate 10 min. at RT. Flick off excess dye. Wash very gently with Barbitol Buffer at 800-1000 rpm for 5 min.
8. Add 5 µl Barbitol Buffer to each well.
9. Read the test results within 10 min. using an inverted phase contrast fluorescence microscope with appropriate filters (e.g. Ploemopak 2.2).

NOTE: If more than one tray is used, stagger the incubations so that results will always be read within 10 min. of staining. In a positive test, the nuclei of the dead cells are stained orange-red by the ethidium bromide. In a negative test, cytoplasm is stained green by fluorescein.

I. Serological 2 Color Fluorescence Microcytotoxicity Test Procedure For Cultured Cells with Unknown HLA Class I Antigens using Standard, Preplated Typing Trays

When all possible antigens are not known (e.g. prenatal paternity tests or cultured endothelial or tumor cells of unknown origin), standard preplated HLA typing trays containing 1 ml serum/well that define all or most HLA A,B,C specificities can be used as follows:

1. Preincubate cells in flasks with IFN-γ (1000-5000 U/ml) for at least 48 hrs.
2. Prepare cells (see section G above) and add 1 µl (1000 cells) to each well of the pre-plated typing trays. Incubate 45 min. at RT.
3. Continue the procedure from step 5 in section H above. Care must be taken that the cells are allowed to settle for 10 min. (alternatively, the trays can be briefly centrifuged for 30 seconds at 2000 rpm) before flicking since they will not, in this case, be attached to the plates.

J. Molecular Techniques for HLA Typing Cultured Cells

Since most cultured cells do not constitutively express class II antigens, molecular methods are the easiest ones to use to establish their class II HLA types. Although typing for class I antigens using molecular methods is now also widely available, many more primer pairs or probes are required to test for unknown class I types. In practice, therefore, the use of molecular typing for identification of class I types for cultured cells is restricted to cases in which the alternative class I types are known. For example, for the case of prenatal diagnosis of the HLA-B linked disease 21-OH-deficiency, we generally identify the 4 alternative HLA-B types by serological tests of the parents of the index case, identify the disease linked HLA-B types by serological typing of the patient, and then use the appropriate primer pairs or probes to identify which HLA-B types the fetus has inherited.

The molecular methods that can be used for HLA typing cultured cells are the same as those used for HLA typing lymphocytes and will not be described here.

K. HLA Typing By Microabsorption of Known Sera: Procedure for Use For Serological Typing Cultured Cells that have Very Poor Viability and/or Cannot be Typed by Cytotoxicity or Molecular Procedures for Other Reasons.

Although absorption is the least elegant and most time-consuming technique for serological HLA typing of cultured cells, it is also the most consistent and reliable procedure. This results from the fact that typical HLA typing sera, which may be operationally monospecific in relation to typing purified T-lymphocytes, also contain a large number of other antibodies which may react with other cells of various types in patterns completely unrelated to their HLA activities38. Absorption can avoid problems with the multiple activities of typing sera because, following the absorption, residual activity in the serum is evaluated with test cells that would fail to react with the non-relevant serum antibodies. Absorption techniques also detect some antigens that appear unusually difficult to detect by cytotoxicity in some individuals' lymphocytes (CYNAP) and some amniotic cells.[39]

Absorption typing of cultured fetal cells can be accomplished with selected antisera representing the 4 possible parental haplotypes, and absorption typing of tumor cell lines from HLA-typed donors can be accomplished with sera representing the donor antigens and several controls. Cell-lines established from donors not characterized for HLA should be tested first by other techniques (e.g., 2 color fluorescence using serum preplated trays) with absorption being used to confirm tentative assignments; otherwise, the number of sera needed would be enormous. The procedure described below for HLA typing by microabsorption is designed to use minimal amounts of cells and sera.

1. Select appropriate typing sera.
2. Prepare a cell suspension and dispense the cells into the total number of microcentrifuge tubes needed for each serum to be used such that 150,000 cells (150 ml) are placed in each tube.
3. Pellet the cells by centrifugation for 1 min. (a Beckman microcentrifuge may be used with a voltage regulator at 1/3 maximum speed.

4. Remove all supernatant medium, first with a Pasteur pipette and then with a 50 ml Hamilton syringe to remove the last few microliters.
5. Add 15 ml of different specific typing sera to each Beckman tube. Note: Typing sera selected for absorption should be preselected and prediluted, if necessary, so that their activity has a titer between 1:2 and 1:4 with all antigen positive test cells ("6" – "8" reactions).
6. Resuspend the cells in each typing serum using a Hamilton syringe and incubate the cells with the sera for 1 hr at RT, mixing every 10 min. on a low-speed Vortex mixer.
7. Centrifuge the cells at full speed (Beckman Microfuge) for 5 min.
8. Remove the absorbed sera with a Hamilton syringe and transfer them to clean tubes.
9. For each serum, centrifuge a sample of unabsorbed serum in the same manner (to remove any debris), and transfer those to clean tubes also.
10. Prepare doubling dilutions of both the absorbed and unabsorbed sera, in microtubes, by placing 7 µl or RPMI 1640 or other medium with 20-30% serum in each of 4 microtubes per serum sample, and sequentially transferring 7 ml of serum into each tube, mixing each time with the transferring Hamilton syringe. This produces dilutions of 1:2, 1:4, 1:8 and 1:16.
11. Plate 5 wells of a microtiter plate with each dilution of absorbed and unabsorbed serum, as illustrated below. Cover and freeze the plates if the "back-tests" are not to be performed immediately.
12. Test each unabsorbed and absorbed sera with 2-5 different cells positive for each relevant class I antigen. These could be unfractionated lymphocytes or separated T-cells. Standard incubation times and complement should be used (see other Procedures).
13. After staining (e.g., with eosin), read the test results with an inverted phase contrast microscope as in the standard lymphocytotoxicity test.

L. Interpretation of HLA Antigens Assignments for 2-Color Fluorescence Cytotoxicity Procedures

1. Antigens can be definitively assigned when the majority of monospecific or multispecific antisera defining that antigen have positive reactions ("4" or greater). Some subtypes of antigens may be assigned by absence of reactions with the alternative subtype or known association with Bw4 vs Bw6 or with particular C locus antigens.
2. If more than 2 antigen assignments per locus result from these criteria, elimination of false/positive assignments can generally be achieved by considering which reactions are weaker and which may result from crossreactive antisera. Example: if antisera defining A2, A11 and A28 are all reactive but the A28 antiserum reactions are much weaker, the assignment of A28 can be considered to be "false/positive", resulting from crossreactivity of the A2 antisera. Alternatively, the microabsorption procedure can be used to confirm assignments.
3. In the absence of more than 2 antigen assignments per locus, antigen assignments based on weak reactions or reactions of a minority of the specific sera can only be considered tentative unless confirmed by repeat testing or unless predicted to be present on the basis of known alternative parental antigens.

M. Interpretation of Absorption Typing Tests

Interpretation: In practice, reduction of activity by more than 50% (see Table 1) occurs only when an antigen is present on the cells. Negative controls, i.e., sera representing specificities known to be absent from the cells from preliminary cytotoxicity tests or because they are absent from both parents, in the case of amniotic fluid cells, should always be included in each absorption experiment.

Table 1. Typical Results in a Microabsorption Experiment

Antigen Positive Test Cells	Absorbed Serum Dilution					Diluent Control
	N	1:2	1:4	1:8	1:16	
#1	6	1	1	1	1	1
#2	4	1	1	1	1	1
#3	8	2	1	1	1	1
#4	6	1	1	1	1	1
#5	6	2	1	1	1	1
	Unabsorbed Serum Dilution					
	N	1:2	1:4	1:8	1:16	
#1	8	8	4	1	1	1
#2	8	6	4	1	1	1
#3	8	8	8	2	1	1
#4	8	8	2	1	1	1
#5	8	8	4	1	1	1

Procedure Notes/Troubleshooting

It is possible to try to treat cultures that appear to be contaminated as follows:

A. **Fungal Infections**
1. At the first (microscopic) sign of contamination, discard the medium, and add fresh medium with 1% Fungizone.
2. Observe the culture and change the medium every day, continuing to use 1% Fungizone until the culture is cleared.
3. If contamination gets worse, discard the culture and decontaminate the incubator and the hood [e.g., with microphene (bacteriocidal) and roccal II (fungicidal)].
4. If fungal contamination occurs frequently, the incubator should be cleaned as above and also disinfected with 10% formaldehyde (avoid inhaling fumes). Leave for 24 hrs, then air out for 7 days before use.

B. **Mycoplasma**

For suspected or confirmed mycoplasma contamination, use 1% anti PPLO Agent (tylocene – Gibco) in the culture medium.

C. **Bacterial Infections**
1. At the first (microscopic) sign of contamination, discard the medium and add fresh medium with twice the normal concentrations of antibiotics.
2. Observe the cultures and change the medium every day using the higher antibiotic concentrations until the culture is cleared.
3. If contamination gets worse, discard culture and decontaminate the incubator and the hood (e.g., with microphene (bacteriocidal) and roccal II (fungicidal).

D. **No Class I reactivity with serologic tests**

For tumor cell lines, if no class I HLA antigens are detected in repeat tests, use a monomorphic monoclonal antibody to class I (e.g., w6/32) antigens with flow cytometry procedures to verify that the gene products are actually present on the cells (some tumor cells lose expression of these antigens[14] even after IFN-γ activation). If not present, you must use a molecular method.

E. **2-Color Fluorescence tests**
1. If the background is too "green" to see "red" (dead) cells, you can quickly rewash the plates once more before rereading.
2. If there are no cells in the wells, you have probably washed or "flicked" the trays too hard. Use a more gentle technique when you repeat the procedure again.
3. If all the cells are either alive or dead, consider changing the complement or incubation times for repeat tests.
4. If all the cells are dead or reactions are weak, consider using a different complement source, or shorter or longer incubation times, respectively, when repeating the test. Use of specifically absorbed complement is also an option.

Acknowledgements

The author acknowledges the significant contribution of all the co-authors of the previous versions of this section:[40-42] C. Callaway, G.L. Grant, C.G. Ioannides, D. Maurer, and S. Sorkin.

References

1. Couillin P, Nicolas H, Boue J and Boue A: HLA typing of amniotic fluid cells applied to prenatal diagnosis of congenital adrenal hyperplasia. Lancet 1:1076, 1979.
2. Pollack MS, Maurer D, Levine LS, New MI, Pang S, Duchon MA, Owens RP, Merkatz IR, Nitowsky HM, Sachs G and Dupont B: Prenatal diagnosis of congenital adrenal hyperplasia (21-hydroxylase deficiency) by HLA typing. Lancet 1:1107, 1979a.
3. Pollack MS, Maurer D, Levine LS, New MI, Pang S, Duchon MA, Owens RP, Merkatz IR, Nitowsky HM, Sachs G and Dupont B: HLA typing of amniotic cells: The prenatal diagnosis of congenital adrenal hyperplasia (21-OH-deficiency type). Transplant Proc 11:1726, 1979b.
4. Pollack MS, Ochs HD and Dupont B: HLA typing of cultured amniotic cells for the prenatal diagnosis of complement C4 deficiency. Clin Genetics 18:197, 1980a.
5. Zoghbi HY, Jodice C, Sandkuijl LA, Kwiatkowski TJ, McCall AE, Huntoon SA, Lulli P, Spadaro M, Litt M, Cann HM, Frontali M, and Terrenato L: The gene for autosomal dominant spinocerebellar ataxia (SCAI) maps telomeric to the HLA complex and is closely linked to the D6S89 locus in three large kindreds. Am J Hum Genet 49:23, 1991.
6. Pollack MS, Schafer IA, Barford D and Dupont B: Prenatal identification of paternity: HLA typing helpful after rape. JAMA 244:1954, 1980b.
7. Gluckman E, Broxmeyer H, Auerbach A, Friedman H, Douglas G, Devergie A, Esperou H, Thierry D, Socie G, Lehn P, Cooper S, English D, Kurtzberg J, Bard J and Boyse E: Hematopoietic reconstitution in a patient with Fanconi's Anemia by means of umbilical-cord blood from an HLA-identical sibling. New Eng J of Med 321:1174, 1989.

8. Pollack MS, Auerbach AD, Broxmeyer HE, Zaafran A, Griffith RL and Erlich HA: DNA amplification for DQ typing as an adjunct to serological prenatal HLA typing for the identification of potential donors for umbilical cord blood transplantation. Hum Immunol 30:45, 1991.
9. Parks LC, Smith WJ and Williams GM: Distinction of allogeneic immunity from tumor specific immunity in man. Surgery 76:43, 1974.
10. Shiku H, Takahasi T, Oettgen HF and Old LJ: Cell surface antigens of human malignant melanoma. II. Serological typing with immune adherence assays and definition of two new surface antigens. J Exp Med 144:873, 1976.
11. Shiku H, Takahasi T, Resnick LA, Oettgen HF and Old LJ: Cell surface antigens of human malignant melanoma. III. Recognition of autoantibodies with unusual characteristics. J Exp Med 145:784, 1977
12. Pollack MS, Livingston PO, Fogh J, Carey TE, Oettgen HF and Dupont B: Genetically appropriate expression of HLA and DR (IA) alloantigens on human melanoma cell lines. Tissue Antigens 15:249, 1980a,
13. Pollack MS, Heagney S and Fogh J: HLA typing of cultured human tumor cell lines: The detection of genetically appropriate HLA A,B,C and DR alloantigens. Transplant Proc 12:134, 1980b.
14. Pollack MS, Heagney SD, Livingston PO and Fogh J: HLA-A,B,C and DR alloantigen expression of forty-six cultured human tumor cell lines. JNCI 66:1003, 1981.
15. Falk K, Rotzschke O, Stevanovic S, Jung G, Rammensee HG. Allelespecific motifs revealed by sequencing of self-peptides eluted from MHC molecules. Nature 351:290, 1991.
16. Rudensky AY, Preston-Hurlburt P, Hong SC, Barlow A, Janeway, Jr.J: Sequence analysis of peptides bound to MHC class II molecules. Nature 353:622, 1991
17. Falk K, Rotzschke O, Deres K, Metzger J, Jung G, Rammensee HG: Identification of naturally processed viral nonapeptides allows their quantification in infected cells and suggests an allele-specific T cell epitope forecast. J Exp Med 174:425, 1991.
18. Pollack MS and Chin-Louie J: Functional properties of the DR antigens expressed on melanoma cell lines as stimulators of primary and secondary proliferative and cytotoxic T-cell responses. Disease Markers 1:147, 1983.
19. Pollack MS, Chin-Louis J and Moshief RD: Functional characteristics and differential expression of class II DR, DS, and SB antigens on human melanoma cell lines. Human Immunol 9:75, 1984.
20. Ioannides, G.G., Fisk, B., Pollack, M.S., Frazier, M.L., Wharton, J.T. and Freedman, R.S.: Cytotoxic T-Cell clones isolated from ovarian tumor infiltrating lymphocytes recognize common determinants on non-ovarian tumor clones. Scand J Immunol 37:413-424, 1993
21. Fisk, B., Chesak, B., Pollack, M.S., Wharton, J.T., and Ioannides, C.G.: Oligopeptide induction of a cytotoxic T lymphocyte response to HER-2/Neu proto-oncogene in vitro. Cellular Immunology 157:415-427, 1994.
22. Fisk, B., Flytzanis, C.N., Pollack, M.S., Wharton, J.T., and Ioannides, C.G.: Characterization of T-cell receptor V-beta repertoire in ovarian tumor reacting CD3+ CD8+ CD4- CTL lines. Scand. J. Immunol. 40:591-600, 1994
23. Mitchell MS: Attempts to optimize active specific immunotherapy for melanoma. Int Rev Immunol 331:348, 1991.
24. Yoshihiko H, Hoon DSB, Park MS, Terasaki PI, Foshag LJ, and Morton DL: Induction of CD4+ cytotoxic T cells by sensitization with allogeneic melanomas being shared or cross-reactive HLA-A. Cellular Immunol 139:411, 1992.
25. McCormick JJ, Yang D, Maher VM, Farber RA, Newman W, Peterson WD and Pollack MS: The HUT series of "carcinogen-transformed" human fibroblast cell lines are derived from the human fibrosarcoma cell line 8387. Carcinogenesis 9:2073-2079, 1988.
26. Maurer DH, Collins WE, Hanke JH, Van M, Rich RR and Pollack MS: Class II positive human dermal fibroblasts restimulate cloned allospecific T cells but fail to stimulate primary allogeneic lymphoproliferation. Human Immunol 14:245, 1985.
27. Maurer DH, Hanke JH, Mickelson E, Rich RR and Pollack MS: Differential presentation of HLA-DR, DQ, and DP restriction elements by interferon-gamma-treated dermal fibroblasts. J Immunol 139:715, 1987.
28. Meade TW, et al: Medical Research Council European Trial of chorion villus sampling. Lancet 337:1491, 1991.
29. Muul LM, Spiess PJ, Director EP, and Rosenberg SA: Identification of specific cytolytic immune responses against autologous tumor in humans bearing malignant melanoma. J Immunol 138:989, 1987.
30. Baker FL, Spitzer G, Ajani JA, Brock WA, Lukeman J, Pathak S, Tomasovic B, Thielvoldt D, Williams M, Vines C and Tofilon P: Drug and radiation sensitivity measurements of successful primary monolayer culturing of human tumor cells using cell-adhesive matrix and supplemented medium. Cancer Res 46:1263, 1986.
31. Ebert T, Bander NH, Finstad CI, Ramsawak RD, and Old LJ: Establishment and characterization of human renal cancer and normal kidney cells lines. Cancer Res 50:5531, 1990.
32. Maurer DH and Pollack MS: The use of gamma interferon to increase HLA antigen expression on cultured amniotic cells used for the prenatal diagnosis of 21-Hydroxylase deficiency. Congenital Adrenal Hyperplasia, Ann. New York Acad Sci, 458:148, 1985.
33. Callaway C, Falcon C, Grant G, Maurer DH, Auerbach AD, Rosenwaks Z and Pollack MS: HLA typing used with cultured amniotic and chorionic villus cells for early prenatal diagnosis or parentage testing without one parent's availability. Human Immunol 16:200, 1986.
34. Maurer DH, Callaway C, Sorkin S and Pollack MS: Gamma interferon induces detectable serological and functional expression of DR and DP but not DQ antigens on cultured amniotic fluid cells. Tissue Antigens 31:174, 1987.
35. Pang, S., Pollack, M.S., Loo, M., Green, O., Nussbaum, R., Clayton, G., Dupont, B. and New, M.I.: Pitfalls of Prenatal Diagnosis of 21-Hydroxylase Deficiency Congenital Adrenal Hyperplasia. Ann. New York Acad. Sci. 458:111-121, 1985
36. Pang, S. Pollack, M.S., Loo, M., Green, O., Nussbaum, R., Clayton. G., Dupont, B. and New, M.I.: Pitfalls of prenatal diagnosis of 21-Hydroxylase deficiency congenital adrenal hyperplasia. J. Clin. Endocrinol Metabol 61:81-97, 1985
37. Kornbluth J, Pollack MS, Fogh J, Carey T and Dupont B: HLA typing of human tumor cell lines: Selection of appropriate typing techniques. Transplant Proc 10:735, 1978.

38. Pollack MS and DuBois D: Possible effects of non-HLA antibodies in common typing sera on HLA antigen frequency data in leukemia. Cancer 39:2348, 1977.
39. Pollack MS, Maurer D, Levine LS, New MI, Pang S, Duchon MA, Owens RP, Merkatz IR, Nitowsky HM, Sachs G and Dupont B: HLA typing of amniotic cells: The prenatal diagnosis of congenital adrenal hyperplasia (21-OH-deficiency type). Transplant Proc 11:1726, 1979.
40. Pollack, M.S., Grant, G.J., Callaway, C., Sorkin, S., and Maurer, D.H.: Class I HLA typing of cultured fetal amniotic fluid, chorionic villus cells and fibroblasts, and other cultured cells (e.g., endothelial cells, tumor cell lines and B cell lymphoblastoid cell lines) In: ASHI Laboratory Manual 3rd Edition, Phelan, D.L., Mickelson, E.M., Noreen, H.S., Shroyer, T.W., Cluff, D.M., Nikaein, A. editors, 1996. I.B. 14.1-14.9.
41. Pollack, M.S., Grant, G.J., Callaway, C., Sorkin S., and Maurer, D.: Establishment and maintenance of cultured fetal amniotic cells, chorionic villus biopsy cells or fibroblasts for use in HLA typing. In: ASHI Laboratory Manual 3rd Edition, Phelan, D.L., Mickelson, E.M., Noreen, H.S., Shroyer, T.W., Cluff, D.M., Nikaein, A. editors, 1996. I.B. 15.1-15.7.
42. Pollack, M.S., and Ioannides, C.G.: Preparation of human non-lymphoid cultured tumor cells for histocompatibility antigen typing. In: ASHI Laboratory Manual 3rd Edition, Phelan, D.L., Mickelson, E.M., Noreen, H.S., Shroyer, T.W., Cluff, D.M., Nikaein, A. editors, 1996. II.B. 5.1-5.9.

Anti-Idiotype Assay

Elaine Reed and Nicole Suciu-Foca

Purpose

The activation and differentiation of T and B lymphocytes require the binding of antigen to their immune receptor. The immune receptors of B cells are immunoglobulin (Ig) and similar to T cells, are clonally distributed and are made up of two-disulfide-bridged glycosylated polypeptide chains: heavy and light chains. Each of the two chains comprises a variable (V) region responsible for the binding of antigen and a constant (C) region. The variable region of both chains of the immune receptors form the combining site (paratope) which permits the recognition of a particular epitope from an unlimited antigenic universe.[1,8]

The light chain of Ig is either Kappa or Lambda chain. The V region of the Kappa and Lambda chains, which represents the B cell receptor, is encoded by two DNA segments, variable (V) and joining (J) genes; the heavy-chain V regions are encoded by three DNA segments, variable (V), diversity (D) and joining (J) genes.

The synthesis of Ig receptor is controlled by two major genetic events: (1) the rearrangement of germ line genes during the differentiation of the B cell lineage and (2) the splicing of the genes. These two events have been estimated to generate 18 billion possible antibodies.

The antigen binding sites (variable regions) of individual antibodies exhibit unique protein structures, which render them immunogenic when injected into another isogeneic, allogeneic or xenogeneic host. Some of the antibodies developed by the secondary host will be specific for the unique determinants of the variable regions of the injected antibody. Such antigenic determinants which distinguish one V domain from another, are referred to as *idiotypes*. Other antigenic markers of antibody molecules which can be identified serologically consist of allotypes or isotypes which distinguish the constant regions of the different heavy-chain classes and light-chain types.[1]

The immune response to any antigen is usually heterogeneous with respect to antibody specificity because different B cells with similar, but non-identical specificities are triggered to proliferate by each antigenic determinant. Consequently, a heterogeneous collection of immunoglobulin molecules are present in each animal's serum.

Since each antibody molecule expresses its unique idiotype, an individual antibody (Ab1) can behave as an antigen and stimulate the generation of the second wave of antibodies (Ab2) which are complementary to the former generation, and referred to as *anti-idiotypes*. In turn, Ab2 can trigger the proliferation of anti-anti-idiotypes (Ab3), and a chain of subsequent generations may result. Since Ab1 is the negative image or the anti-image of the antigen, Ab2 has also been termed internal image of the antigen. Similarly, Ab3 can represent the internal image of Ab1, and Ab4 the internal image of Ab2. The connections between individual antibodies in this network of interactions can be either open ended or closed. In addition to idiotypes which are situated within the binding region (paratope) of the antibody molecule, there are idiotypes which lie outside the antigen combining site and thus induce a cascade of anti-idiotypic antibodies which do not recognize the original immunizing antigen.

The idiotype network plays an important role in the regulation of immune responses and may account for cyclical variations in the level of reactive antibodies that sometimes occurs.

A large number of experimental observations demonstrate that idiotypes behave as self and foreign antigens at the same time. Certain idiotypes become dominant as a consequence of recognition by regulatory T cells, and serve as markers of V germ line genes. These idiotypes can be shared by antibodies with distinct specificities, and by individuals of the same or different species. They are called regulatory idiotypes and are presumed to serve important biological functions. Antibodies induced by the same antigen in different individuals can also share a public, or crossreactive idiotype (Idx). Interstrain and interspecies Idx are expressed not only by Abl but also by anti-idiotypic antibodies.

The discovery of Idx, namely antigenic specificity shared among antibodies of several individuals against the same antigen, represented a crucial step in research on the regulatory role of idiotypes within the immune system. The extensive crossreactive idiotypy of antibodies specific for antigenic determinants of anti-Ig antibodies [i.e., anti-Id, anti-allotype and rheumatoid factors (RF)] indicate that these responses are highly conserved and encoded by germ-line variable region genes. Although human anti-HLA antibodies are heterogeneous, they nevertheless share Idx which are recognized by naturally occurring anti-Id antibodies.

In previous studies we have shown that anti-idiotypic antibodies specific for anti-HLA antibodies are present in sera from individuals who have been allosensitized to HLA antigens by transfusions, transplantation or pregnancy.[2,3,9-23] We found that in presensitized recipients the presence of Ab2 correlates positively with allograft survival, while the presence of Ab3 is predictive of early graft failure.[12] Identification of such antibodies is therefore, important in crossmatching procedures.

The principle of the assay used for assessing the presence of anti-anti-HLA (Ab2) antibodies resides in determining whether sera, which are negative for anti-HLA antibodies, are endowed with the capacity of blocking specifically, certain anti-HLA antibodies developed by the same or by other individuals. For this, equal amounts of serum tested for Ab2 are added to serial two-fold dilutions of Ab1 (anti-HLA serum). Sera obtained from individuals who have not been exposed to allogeneic HLA antigens are used as controls. The mixtures are incubated at 4° C for one hr and then tested for anti-HLA activity, using the complement-dependent lymphocytotoxicity assay. Binding of Ab1 to Ab2 will result in inhibition

of Ab1 and, subsequently, the titer of anti-HLA antibodies (Ab1) will be decreased. Thus, the titer of the anti-HLA serum will be at least one fold or two fold lower in the presence of Ab2 then in its absence. The specificity of the reaction is determined using a set of antisera containing antibodies against the same or other HLA antigens. The blocking activity of a serum containing anti-idiotypic antibodies can be ascertained in the autologous system, i.e., using Ab1 positive sera from the same individual, or in the homologous system, i.e., using sera from other individuals producing the same anti-HLA antibody.

For example, patient A showed anti-HLA-A26 antibodies (Ab1) in a serum obtained one month following a blood transfusion. The titer of the anti-A26 antibodies was 1/16. Since no anti-A26 antibodies were detected in subsequent bleedings collected from patient A, and since a sibling carrying the A26 antigen is being considered as a potential donor, we want to determine whether the patient has developed anti-anti-HLA-A26 antibodies. For this, equal amounts of sera tested for Ab2 are added to serial two-fold dilutions of autologous Ab1. The Ab1-Ab2 mixtures are incubated at 4° C for one hr, and tested for cytotoxic activity against A26 positive target cells. If only the 1/2-1/4 dilutions of Ab1 are positive in the presence of the patient's Ab1 negative sera, the possibility that these sera contain Ab2 should be considered. Since anti-idiotypic antibodies are characterized by their exquisite specificity, it remains to be shown that the blocking activity of patient's A sera is specific for anti-HLA-A26 antibodies. The specificity of the blocking effect should be investigated by determining whether the negative sera from patient A, also inhibit anti-A26, and anti-A10 antibodies from other individuals but not antibodies against other HLA antigens which share no public determinants with the A26 antigen.

If other sera with anti-HLA-A26 antibodies are also blocked, the conclusion can be drawn that the anti-idiotypic antibodies present in patient A's serum recognize an HLA-A26-related Idx. However, if blocking occurs only in the autologous system, a private idiotype may be involved. If rather than inhibiting the cytotoxic activity of antibodies specific for a distinct HLA antigen, the serum blocks any alloantisera, including sera with multispecific antibodies, factors other than anti-idiotypic antibodies are likely to be involved.

Specimen

One ml of patient's serum containing anti-HLA antibodies, one ml of serum to be tested for Ab2 activity and 10^7 lymphocytes from the transplant donor.

A lymphocyte suspension prepared by any method that provides viable cells (≥95%). See cell isolation chapters for methods.

Unacceptable Specimen

Sera containing lymphocytotoxic autoantibodies should not be tested unless treated with Dithiothreitol (DTT) to remove non-HLA, IgM cytotoxins.

Lymphocyte suspensions with less than 95% viability or excessive contamination with monocytes, granulocytes or red cells.

Reagents

1. Modified McCoys 5A medium supplemented with 0.5% fetal calf serum (FCS). Store at 2-8° C.
2. C-FDA:
   Dissolve 0.1 mg 5 and 6 Carboxyfluorescein diacetate in 0.2 ml acetone in a polypropylene tube. Store at -20° C.
3. Ethidium Bromide (EB)
   Dissolve 100 mg EB in 10 ml EDTA. Store at 4° C. Caution: EB is a potential carcinogen. Use disposable gloves when handling this material.
4. Anti-HLA sera
   A comprehensive set of operationally monospecific reagents which recognize distinct HLA antigens (correlation coefficients >0.7).
5. Complement
6. Immunogmagnetic beads coated with murine anti-human HLA class I antibodies and murine anti-human HLA class II antibodies for depletion of soluble HLA antigens.
7. Beckman tubes (0.2 ml)
8. Terasaki microtest trays (60 or 72 well)
9. Light mineral oil

Instrumentation

1. Same as used in conventional HLA serology.
2. Centrifuge used for cell and serum isolation
3. Fluorescence microscope
4. Cell plating/dotting instruments and/or Hamilton syringes for adding cells and sera to microtest plates.

Calibration

Controls

1. Negative Control. Most laboratories use a serum obtained from a healthy, non-transfused donor. The serum must be screened and found negative for cytotoxic activity. The negative control is used to determine the viability of the cells used in the lymphocytotoxicity assay. The negative control well should have a viability >80%.
2. Positive Control. Most laboratories use anti-lymphocytic serum, serum from a highly sensitized person or a pool of sera from highly sensitized individuals, or murine monoclonal anti-HLA antibodies. The positive control is used to demonstrate that all the reagents required for the lymphocytotoxicity reaction are present in the test. The positive control well should have a viability of less than 20%.
3. B Cell Control. The B cell control is included in the assay to determine the percentage of HLA-class II positive B cells. Most laboratories use as a B cell control a murine monoclonal antibody directed against a monomorphic determinant on HLA class II antigens.

Quality Control

Reagent Quality Control: All new lots of reagents including media, complement, anti-HLA antisera, monoclonal antibodies, and controls must undergo routine quality control testing.

Procedure

Selection of Cases

1. Examine the patient's history of antibodies against HLA antigens. Select patients who have formed antibodies against a distinct HLA specificity and showed a loss of the respective antibody in one or more subsequent bleedings.
2. Screen both the positive and the negative sera in 1/1 to 1/256 dilutions using an informative panel of T and B lymphocytes. The case is informative only if the anti-HLA serum has a titer of at least ½ and the putative Ab2 serum shows no activity at any dilution.
3. Confirm the absence of antibodies in the "negative" serum (putative Ab2) by a method which permits the detection of non-complement binding antibodies such as complement mediated lymphocytotoxicity in the presence of goat anti-human Ig antibodies[6] or indirect immunocytofluorometry.[12,13] Non-complement fixing antibodies will compete with complement fixing (cytotoxic) antibodies for binding to target cells. Sera containing such antibodies cannot be used in competition assays aimed to detect anti-idiotypic antibodies, unless the anti-HLA antibodies are absorbed. If absorption of anti-HLA antibodies is performed, the IgG fraction of the serum must be obtained and used in anti-idiotypic assays.[9] During absorption of anti-HLA antibodies on B-lymphoblastoid cell line (BLCL) or on pooled platelets, soluble HLA antigens are released from the cells into the serum. Since soluble HLA antigens inhibit the cytotoxic activity of anti HLA antibodies, absorbed sera cannot be used unless depleted of HLA antigens and the IgG fraction of the serum obtained.
4. Each serum used in the study must be depleted of soluble HLA antigens.[22,237] The depletion of soluble HLA antigens is performed using immunomagnetic beads. Use Dynabeads HLA Cell Prep II (Dynal Inc., Great Neck, N.Y.) for depletion of HLA class II antigens. Incubate 150 µl of serum with 50 µl of Dynabeads (containing 4 x 10^8 beads/ml). After one hr of incubation at 4° C with continuous mixing, remove the beads using a magnet. Use Magnisort -M chromium dioxide particles (Dupont Co., Wilmington, DE) for depletion of HLA class I. Coat the particles with murine monoclonal antibody (MoAb) B9.121 (Pel Freeze, Brown Deer, WI) which reacts with a common epitope of all HLA class I molecules. Coating is accomplished by incubating 1 ml of Magnisort-M with 3 ml of purified MoAb B9.121 at 7 mg of IgG/ml for 1 hr at 4° C. Collect the beads using a magnet, wash them 3 times in PBS and resuspend them in 1 ml of PBS. Use the B9.121 coated beads for HLA class I antigen depletion. For this, add 10 µl of coupled beads to 500 µl of serum, and incubate for one hr at 4° C with continuous, gentle mixing. Remove the beads using the magnet and collect the HLA class I antigen depleted serum. The completion of depletion can be checked by determining whether the antigen depleted serum inhibits the binding of murine anti-HLA MoAb to human lymphocytes.
5. For each individual whose sera are examined for Ab2 we must identify a healthy donor of normal human serum who will be used as a negative control in parallel blocking assays. The control serum donor must be an individual who has not been exposed to allogeneic HLA antigens by pregnancy, transfusion or transplantation. This serum should be depleted of soluble HLA antigens prior to use.

Inhibition of Lymphocytotoxicity

1. Centrifuge anti-HLA sera (Ab1), HLA-antigen depleted test sera (Ab2) and control sera (C) for 10 min at 7000 x g to remove any precipitate.
2. Prepare serial dilutions of Ab1 in 0.5% McCoys medium using 0.2 µl Beckman tubes. Dilutions should range from ½ to the highest dilution known to result in complete lysis of target cells.

3. Plate 1 µl of each dilution of Ab1 in individual wells of Terasaki microtest trays, containing 2-5 µl of light weight mineral oil, according to a pre-established protocol. For each target cell to be used, two plates have to be prepared: one for testing the blocking activity of the test serum and the other for testing a negative control serum.
4. Add to each dilution of Ab1, 1 µl of Ab2. In the parallel tray add 1 µl of negative control serum to each dilution of Ab1.
5. Incubate the plates for 1 hr at 4° C.

Lymphocytotoxicity

1. Prepare purified T and B lymphocyte suspensions using cell separation methods which permit maximal enrichment of T or B lymphocyte populations. We routinely use the nylon wool column technique[5] to purify B lymphocytes. The nylon wool adhering cells represent the Ia positive population used for detection of anti-HLA-DR antibodies. The non-adhering cells are mostly T lymphocytes.
2. Stain the lymphocyte populations with C-FDA using the method of Bodmer et. al.[4] as follows.
   a. Resuspend the lymphocytes (0.1 to 1 x 10^7 cells) in 0.8 ml of RPMI-1640 medium.
   b. Add 0.2 ml of C-FDA (0.1 mg) to the cells and incubate at room temperature (RT) for 15 min.
   c. Wash the cells twice, and establish the lymphocyte viability and cell count using a 0.2% Trypan blue solution in RPMI-1640.
3. Thaw anti-idiotype assay trays immediately before using. All assays should be performed in duplicate.
4. Add 1 µl of target lymphocyte suspension containing 2-3 x 10^6 cells/µl to each well.
5. Incubate cells and sera for one hr at 22° C.
6. Add 5 µl of pretested rabbit HLA-DR complement to trays containing B lymphocyte suspensions and 5 µl of pretested rabbit HLA-A,B,C complement to trays containing T lymphocyte suspensions.
7. Incubate trays containing the mixture of cells, sera and complement for one hr at 22° C.
8. Add 2 µl of EB to each well.
9. Read trays using an inverted fluorescence microscope. Living cells convert C-FDA to carboxyfluorescein and fluoresce green and dead cells will lose C-FDA, be permeated by EB and fluoresce red.

   Note: Dye exclusion with eosin or trypan blue may also be used.
10. Score reactions using the NIH scoring technique (see HLA Typing by Lymphocytotoxicity method).

Calculations

The final titer of each serum is considered to be the highest dilution killing 75% of target lymphocytes. The

$$\% \text{ inhibition} = \frac{Ab1{:}C - Ab1{:}Ab2}{Ab1{:}C} \times 100$$

percent inhibition of lymphocytotoxic activity of Ab1 by Ab2 is calculated from the formula:

Key: Percent killing of target lymphocytes in the following sera:

Ab1:C Ab1 diluted in medium or control serum
Ab1:Ab2 Ab1 diluted in Ab2 test serum

Results

A serum is considered to contain Ab2 if it specifically blocks the cytotoxic activity of Ab1. This is demonstrated by a decrease in the titer of Ab1 when mixed with the Ab2 test serum, as compared with the titer when the same Ab1 is mixed in a control serum. If a serum contains blocking activity it is important to demonstrate that it is indeed idiotype specific by testing other autologous or homologous sera containing Ab1 to the same specificity. Also, Ab1 reacting with a different HLA specificity should be included since such antibodies should not be blocked by the same Ab2. A serum contains anti-idiotypic antibodies (Ab2) against anti-HLA antibodies (Ab1) if it blocks specifically antibodies reactive with a distinct HLA antigen.

In addition to antibodies which block the cytotoxic activity of Ab1 we have found that certain sera contain antibodies which augment or potentiate the cytotoxic activity of Ab1. This is demonstrated by an increase in the titer of Ab1 when mixed with the Ab2 test serum, as compared to the titer of Ab1 diluted in medium or in sera from individuals who have not been exposed to allogeneic HLA antigens. Increases in the titer of cytotoxic antibodies can be caused by the presence of subthreshold levels of anti-HLA antibodies in a seemingly negative serum. The existence of weakly binding or non-complement binding anti-HLA antibodies can be investigated by immunocytofluorometry studies or using the anti-immunoglobulin test described by Fuller et. al.[6] If the serum shows no anti-HLA antibodies by any of these procedures, its Ab1 potentiating activity may be caused by anti-anti-anti-HLA antibodies, or Ab3, which mediate the release of Ab1 from the Ab1-Ab2 complexes present in the Ab1 positive sample. Like Ab2, Ab3 should be idiotype specific. Therefore Ab3 should potentiate Ab1 of the same specificity yet not Ab1 which are specific for different HLA antigens.

Procedural Notes

1. Non-specific blocking factors.
   There are several factors which can account for the blocking activity of human sera. These factors include the following:
   a. Soluble HLA antigens. Human sera contain soluble HLA antigens which inhibit the cytotoxic activity of anti-HLA alloantibodies.[7] Thus, all sera must first be depleted of soluble HLA antigens prior to use in the anti-idiotypic assay.[22,23]
   b. Rheumatoid Factors (RF). RF are immunoglobulins which bind to the Fc region of IgG and IgM antibodies. The presence of RF can cause non-specific blocking of Ab1. RF can be identified using commercially available kits.
   c. Non-complement fixing antibodies. If non-complement fixing antibodies are present in a serum which is tested for Ab2 activity they may compete with Ab1 for binding to the HLA antigens of the target lymphocytes. For this reason, each serum tested for Ab2 must be crossmatched by flow cytometry and/or by the antiglobulin assay if it exhibits blocking activity in the anti-idiotype assay. If non-complement fixing Ab1 are detected, the serum must be absorbed on platelets and BLCL to remove residual Ab1. During the absorption procedure, HLA antigens from cell membranes are released in the serum. Since soluble HLA antigens will render the serum inhibitory, they must be depleted from the absorbed serum prior to use in the anti-idiotype assay. This can be accomplished by incubating the serum for 60 min with magnetic beads coated with monoclonal antibodies specific for HLA-Class I or Class II antigens.[22,23] The IgG fraction of the HLA depleted serum should then be obtained and tested for anti-idiotypic activity.[9]
2. Incorrect titration of Ab1.
   The most common problem encountered in this assay is the failure to observe a reproducible, linear titration curve when Ab1 is diluted in media or serum. This error is usually caused by inaccurate pipetting or insufficient mixing of Ab1 with the test serum used as a diluent. When cells from different individuals expressing the same antigen are used as targets for titering an alloantiserum, differences in the final titer are also frequently observed. Such differences can be caused by the level of expression of HLA on the membrane of target cells, or by the affinity of the antibodies for a certain epitope and/or by the sensitivity to lysis of the target cells used. It is advisable to use highly purified T or B cell suspensions in the assay and to compare results obtained on cells cryopreserved under similar conditions.
3. Failure of Ab2 to block AB1 from different individuals.
   Anti-idiotypic antibodies from one individual may inhibit Ab1 from one, but not from another individual. Such differences are expected to occur, since the V gene repertoire used for the generation of anti-HLA antibodies may differ from one individual to another. Furthermore, given the polyclonal nature of anti-HLA antibodies present in alloantisera, blocking of only some but not of all idiotypes, may occur. The unblocked antibodies may be sufficient to cause lysis of the targets, thus obscuring Ab1-Ab2 reaction.

Limitation of Procedure

Soluble HLA antigens, rheumatoid factors and non-complement fixing anti-HLA antibodies can interfere with the detection of anti-idiotypic antibodies. Please see Procedure notes for a detailed explanation.

References

1. Bona CA: *Modern Concepts in Immunology*, Volume II. Wiley-Interscience, New York; 1987.
2. Bonagura V, Rohowsky-Kochan C, Reed E, Ma A, McDowell J, King DW, Suciu-Foca N: Brief definitive report: perturbation of the immune network in herpes gestationis. Hum Immunol 15:211-219, 1986.
3. Bonagura VR, Ma A, McDowell J, Lewison A, King DW, Suciu-Foca N: Anti-clonotypic autoantibodies in pregnancy. Cellular Immun 108:356-365, 1987.
4. Bodmer W and Bodmer J: Cytofluorochromasia for HLA-A,B,C and DR Typing. In: *Manual of Tissue Typing Techniques*; Ray J, Hare, ed.; National Institute of Allergy and Infectious Diseases, NIH, Bethesda, p 46-54, 1979.
5. Danilovs J, Terasaki PI, Park MS, Ayoub G: B lymphocyte isolation by thrombin nylon wool. In: *Histocompatibility Testing* 1980; P Terasaki ed.; UCLA Tissue Typing Laboratory, Los Angeles; p 287-288, 1990.
6. Fuller T, Phelan D, Gebel H, Rodey G: Antigenic specificity of antibody reactive in the antiglobulin-augmented lymphocytotoxicity test. Transplantation 34:24-29, 1982.
7. Guencheva G, Scholz S, Schiessl B, Albert ED: Soluble HLA antigens in normal human immunglobulin preparations. Tissue Antigens 19:198-201, 1982.
8. Hood L, Weissman I, Wood WB, Wilson JH: *Immunology*. The Benjamin Cummings Publishing Company, Inc., California, 1984.
9. Reed E, Bonagura V, Kung P, King DW, Suciu-Foca N: Anti-idiotypic antibodies to HLA-DR4 and DR2. J Immunol 131(6):2890-2894, 1983.
10. Reed E, Rohowsky-Kochan C, and Suciu-Foca N: Analysis of 9W antisera detecting DR4 and DR2 associated epitopes by use of anti-idiotypic antibodies. In: *Histocompatibility Testing 1984*, Albert ED and Baur WR eds.; Springer-Verlag, New York; p 422-424, 1984.

11. Reed E, Hardy M, Lattes C, Brensilver J, McCabe R, Reemtsma K, and Suciu-Foca N: Anti-idiotypic antibodies and their relevance to transplantation. Transpl Proc 17:735-738, 1985.
12. Reed E, Hardy M, Benvenisty A, Lattes C, Brensilver J, McCabe R, Reemtsma K, King DW, Suciu-Foca N: Effects of anti-idiotypic antibodies to HLA on graft survival in renal-allograph recipients. New Eng J Med 316:1450-1455, 1987.
13. Reed E, Beer AE, Hutcherson H, King DW, and Suciu-Foca N: The alloantibody response of pregnant women and its suppression by soluble HLA antigens and anti-idiotypic antibodies. J Reprod Immunol 20:155-128, 1991.
14. Reed E, Ho E, Cohen DJ, Marboc C, D'Agati V, Rose EA, Hardy M, Ramey W, and Suciu-Foca N: Anti-idiotypic antibodies specific for HLA in heart and kidney allograft recipients. J Immunol Res 12:1-11, 1993.
15. Rohowsky C, Reed E, Suciu-Foca N, Kung P, Reemtsma K, King DW: Inhibition of MLC reactivity to autologous alloactivated T-lymphoblasts by sera from renal allograph recipients. Transplant Proc 15:1761-1763, 1983.
16. Suciu-Foca N, Reed E, Rohowsky C, Kung P, King DW: Anti-idiotypic antibodies to anti-HLA receptors induced by pregnancy. Proc Natl Acad Sci 80:830-834, 1983.
17. Suciu-Foca N, Reed E, Rohowsky-Kochan C, Popovic M, Bonagura V, King DW, Reemtsma K: Idiotypic network regulations of the immune response to HLA. Transplant Proc 17:716-719, 1985.
18. Suciu-Foca N, King DW, Reemtsma K, Kohler H: Autoimmunity and self-antigens. Concepts in Immunopathology 1:173-189, 1985.
19. Suciu-Foca N, Reed E, King DW, Lattes C, Brensilver J, McCabe R, Benvenisty A, Hardy M, Reemtsma K: Idiotypic network regulations of allograph immunity. In: *Transplantation and Clinical Immunology*: Touraine JL, ed.; Elsevier Science Publishers, P 35-44, 1985.
20. Suciu-Foca N, Reemtsma K, King DW: The significance of the idiotypic anti-idiotypic network in humans. Transplant Proc 18:230-234, 1986.
21. Suciu-Foca N and King DW: The biological significance of anti-idiotype autoimmune reactions to HLA. In: *Biological Adaptations of Anti-Idiotypes*; CA Bona ed.; CRC Press, Inc., Boca Raton, Vol. 2:149-163, 1988.
22. Suciu-Foca, Reed E, D'Agati VD, Ho E, Cohen DJ, Benvenisty AI, McCabe R, Brensilver JM, King DW and Hardy MA: Solule HLA-antibodies and anti-Idiotypic antibodies in the circulation of renal transplant recipients. Transplantation 51:594-601, 1991.
23. Siciu-Foca N, Reed E, Marboe C, Yu Ping Xi, Sun Yu-Kai, Ho E, Rose E, Reemtsma K and King DW: Role of anti-HLA antibodies in heart transplantation. Transplantation 51:716-724, 1991.

Lymphocyte Crossmatch: Extended Incubation and Antiglobulin Augmented

Patti A. Saiz and Cynthia E. Blanck

Purpose

The purpose of the lymphocyte *crossmatch* is to detect the lymphocytotoxic antibodies specific to a potential donor (i.e., allogeneic crossmatch) or self (i.e., autologous crossmatch.) A forward crossmatch is used with serum from a prospective recipient (or donor for reverse crossmatch) and the target cells are the mononuclear cells of the potential donor (or recipient for reverse crossmatch). The target cells used may be unseparated or separated into specific subsets such as T cell lymphocytes, B cell lymphocytes, monocytes, etc.

Specimen

1. Acceptable Specimens
   a. The peripheral venous blood should be routinely used as the source of serum and lymphocytes for crossmatch testing.
      1) Peripheral blood for serum is drawn into a 10 ml red top (clot tube without additives).
      2) Peripheral blood for lymphocyte isolation is drawn into yellow top (ACD-A) vacutainer tubes. Green top (sodium heparin) vacutainer tubes do not preserve cells as well as ACD does and may only be used in case of emergency. For most patients, one to three (1-3) ACD tube(s) will be sufficient for a lymphocyte crossmatch. If the patient has an abnormal white blood cell count (i.e., total WBC, percent lymphocytes or differential), the laboratory should be notified before drawing the patient to verify how many tubes of blood will need to be drawn.
   b. Specimen tubes should be labeled and logged in according to the laboratory's procedure.
   c. All blood will be maintained at room temperature and transported to the laboratory as soon as possible, preferably arriving no later than 24 hours after being drawn.
2. Unacceptable Specimens
   a. Specimens more than 24 hours old. Blood more than 24 hours old is undesirable, because cells may have reduced viability. Cells can be isolated and tested for viability. Viability must be at least 80% to perform serological typing.
   b. A specimen that has been frozen or refrigerated.
   c. A specimen that has been drawn in a tube not listed as "acceptable" in this procedure.
   d. A clotted specimen collected in an anticoagulant tube.
   e. An unlabeled specimen.
   f. A grossly hemolyzed specimen.
   g. RESOLUTION: When an unacceptable specimen is received, document the circumstances according to QA (Quality Assurance) protocol and notify (1) the Supervisor and (2) appropriate personnel to request re-collection as soon as possible.

Reagents and Supplies

1. RPMI (plain, for washing cells on tray).
2. 2.5% FBS/RPMI (for diluent and cell suspensions).
3. Proper Controls -- The following controls can be purchased, aliquoted into suitable tubes and stored frozen at -70° C (or colder) until needed.
   a. ALS (Anti-Lymphocyte Serum) positive T and B cell control serum.
   b. ABS (Anti-B cell Serum) positive B Cell control serum.
   c. PHS (Pooled Human Serum) negative control.
4. Complement.
5. AHG (goat IgG anti-human globulin, anti-kappa light chain – anti-Fab type).
6. Stain (FluoroQuench™ AO/EB Stain-Quench Reagent or equivalent).
7. Mineral oil.
8. 60, 72, or 96-well polystyrene microtiter trays with optically clear bottoms. The inner surface of each well is wettable.

Instrumentation/Special Equipment:

1. Fluorescent inverted microscope. Maintenance will be performed according to instructions in the equipment maintenance manual.
2. Light box.
3. Micropipettes to deliver 1 µl and 5 µl amounts.
4. Timer.
5. Centrifuge with buckets capable of holding trays.
6. Pipet dispenser filled with mineral oil.

Calibration

Calibration of equipment (i.e., microscope, micropipettes, timer, centrifuge, pipet dispenser) should be performed per manufacturers recommendations. In particular, the centrifuge should be calibrated for the speeds to be used in this procedure.

Quality Control

Known positive and negative controls must be run with each crossmatch tray. Standard reagent QC procedures should be followed and must be documented.

Procedure

Crossmatch Conditions

1. Types
   a. Lymphocyte Crossmatch: Unseparated lymphocytes (PBLs) or T-lymphocytes are plated and incubated at the temperatures 4° C, 22° C (room temperature), and 37° C for the pre-complement incubation.
   b. T and B Crossmatch: T and B lymphocytes are plated. The T and B cells are incubated at 4° C, 22° C (room temperature), and 37° C for the pre-complement 30 minute incubation. The B cell 4° C incubation may be omitted if desired.
   c. B Cell Crossmatch: B cells are plated and incubated at 22° C (room temperature) and 37° C for the pre-complement incubation.
2. Categories – Both "Lymphocyte" and "T and B" Crossmatch can include the following crossmatch categories:
   a. Autologous: Patient sera vs. patient cells (or donor sera vs. donor cells.)
   b. Forward: Patient sera vs. donor cells.
   c. Reverse: Donor sera vs. patient cells.

Cell Preparation

1. For a T-cell crossmatch, prepare a lymphocyte suspension (either PBL, T-enriched cells or T Dynal cells) in 2.5% FBS/RPMI at a concentration of 3 x 10^6/ml (see lymphocyte isolation procedure.)
2. For a B-cell crossmatch, prepare a B-cell enriched lymphocyte suspension (B Dynal cells or equivalent) in 2.5% FBS/RPMI at a concentration of 2 x 10^6/ml (see lymphocyte isolation procedure.)
3. PBLs (peripheral blood lymphocytes) or Unseparated Lymphocytes – Acceptable sources include:
   a. Ficolled lymphocytes from peripheral blood.
   b. A cell preparation prepared after B immunogenetic bead depleted supernatant has been ficolled (usually a mini-ficoll prep).
   c. T immunogenetic bead prepared cells or equivalent.
   d. Lympho-Kwik Mononuclear prepared cells.
4. T Cells – Acceptable sources include:
   a. Ficolled lymphocytes from peripheral blood (predominantly T cells).
   b. A cell preparation prepared after B immunogenetic bead depleted supernatant has been ficolled (usually a mini-ficoll prep).
   c. T immunogenetic bead prepared cells or equivalent.
   d. Lympho-Kwik T prepared cells.
5. B Cells – Acceptable sources include:
   a. B immunogenetic bead prepared cells or equivalent.
   b. Lympho-Kwik B prepared cells.
6. The laboratory does not recommend nylon wool separation for T and B cells.

Tray (Sera) Preparation

Note: Sera are chosen according to type of transplant being performed (see Transplant Protocol Section). All serum is dispensed into disposable polystyrene trays that have 60, 72, or 96 wells per tray. The 72 well tray is sufficient for standard crossmatches.

1. Whole blood that has been obtained in a red top tube from the individual to be tested is allowed to clot. Centrifuge clot tube for five minutes at 2500 RPM without brakes. Serum aliquots are stored frozen at -70° C.

2. Prepare Crossmatch Tray Format worksheets for trays (see example at end of chapter). Determine layout according to the temperatures and techniques to be used
3. Label trays with XM#, tray #, and pre-complement incubation temperature as a minimum. Patient name (or ID#), tech initials and date tested may also be added if desired.
4. Add 5 µl of mineral oil to each well with a pipet dispenser. The mineral oil will prevent evaporation of the sera. After oiling trays, store them at 4° C until the sera is ready to be added.
5. Using 2.5% FBS/RPMI media as the diluent, serum from the prospective recipient (or donor for reverse crossmatches) is diluted serially from neat (i.e., 1:1 or no dilution) to 1:8. Negative control, positive control, and test sera are diluted in the same manner. Minimum amounts of sera can be diluted using a 50 µl, 80 µl or 100 µl Hamilton syringe using dilution technique described in Procedure Note #3.
6. Add 1 µl of each dilution to correct well per tray worksheet (see Procedure Note #4).
7. Check trays on light box to be sure that there are sera in each well under the oil.
8. The trays are now ready for addition of cells and testing with the NIH or AHG Crossmatch Technique. Store trays at 4° C and use within 24 hours, or store in a sealed container (e.g., sealed plastic bag) at -70° C freezer until used.
9. T cell crossmatches are commonly run at 4° C, 22° C, and 37° C. B cell crossmatches are usually run at 22° C and 37° C. Autologous crossmatches are often run at 22° C only.

NIH Crossmatch Technique – Extended Incubation

Note: This technique can be used on unseparated lymphocytes, T cells or B cells.

1. Warm trays to room temperature just before using. If trays were frozen, visually verify the wells contain sera and allow them to remain at room temperature until antisera is completely thawed (approximately 5-15 minutes)
2. Verify that tray labels matches the Crossmatch Tray Format sheet, the cells and the sera being tested.
3. Mix the lymphocyte suspensions thoroughly. Check concentration and viability. The cell suspensions should be at a concentration of 2 x 10^6/ml and 80% viable.
4. Add 1 µl of the appropriate cells to each well according to the Crossmatch Tray Format. Be careful not to touch the sera already in the well with the tip of the pipetting needle (see Procedure Note #4).
5. Be sure that the cell suspension has mixed well with the HLA sera in each well. That may be done by gentle shaking of the tray, by static mixing with the high frequency generator, or by using a pin point to bring the cell suspension droplet together with the HLA serum droplet. Be sure to clean the pin before going to the next well.
6. Incubate cells and sera for 30 minutes at the appropriate temperature (4° C, 22° C [i.e., room temperature] or 37° C).
7. Following incubation, add 5 µl of appropriate complement to each well and incubate T cells for 55 minutes (B cells for 45 minutes) at room temperature (22° C).
8. Following complement incubation, add 5µl of Stain to each well. Store tray at 4° C in the dark until read. Trays are routinely read immediately or at least within 24 hours, but, when stored at 4° C in the dark with over 90% cell viability, trays may be readable up to 48 hours.

Antiglobulin Crossmatch Technique (AHG) – Antiglobulin Augmented

Note: AHG technique is a method to enhance sensitivity of the T cell crossmatch and is not routinely used for the B cell crossmatch. The T cell enriched population should be 80% T cells or greater. Ficolled, unseparated spleen lymphocytes must be enhanced for T cells before using in the AHG technique. The spleen usually has only 50%-60% T lymphocytes and 40%-50% B lymphocytes. T-cell enriched, ficolled peripheral blood lymphocytes, perfused node lymphocytes or B-cell depleted lymphocytes are all acceptable cell preparations for the AHG technique.

1. Warm trays to room temperature just before using. If trays were frozen, visually verify the wells contain sera and allow them to remain at room temperature until antisera is completely thawed (approximately 5-15 min.)
2. Verify that tray labels matches the Crossmatch Tray Format sheet, the cells and the sera being tested.
3. Mix the lymphocyte suspensions thoroughly. Check concentration and viability. The cell suspensions should be at a concentration of 2 x 10^6/ml to each well according to the Crossmatch Tray Format.
4. Be careful not to touch the sera already in the well with the tip of the pipetting needle (see Procedure Note #4).
5. Be sure that the cell suspension has mixed well with the HLA sera in each well. That may be done by gentle shaking of the tray, by static mixing with the high frequency generator, or by using a pin point to bring the cell suspension droplet together with the HLA serum droplet. Be sure to clean the pin before going to the next well.
6. Incubate cells and sera for 30 minutes at the appropriate temperature (4° C, 22° C [i.e., room temperature] or 37° C).
7. Following incubation, wash trays three times using RPMI for the first two washes and 2.5% FBS/RPMI for the third wash.

   Note: During wash steps prepare complement and dilute AHG to be ready for next step immediately at the end of the last wash step flick!

   a. Add 10 µl of RPMI (2 clicks on 5 µl micropipettor) to each well.
   b. Centrifuge trays for one minute at 1000 RPM.
   c. "Flick" tray over sink to remove wash solution. (To properly flick a tray, hold an uncovered tray by its sides and in one, smooth motion snap wrist down for an even removal of solution without carryover into other wells.).

d. For the second wash step, add 10 µl of RPMI to each well and centrifuge again for one minute at 1000 RPM. Flick trays evenly.
e. For the third wash step, add 10 µl of 2.5% FBS/RPMI to each well and centrifuge again for one minute at 1000 RPM. Flick trays hard and evenly to remove media and leave cells in bottom of wells.

8. Add 1 µl of properly diluted anti-human globulin (AHG) to each well.
9. Exactly one minute later, add 5 µl of appropriate complement.
10. Incubate for 55 minutes at room temperature.
11. Following complement incubation, add 5 µl of Stain to each well. Store tray at 4° C in the dark until read. Trays are routinely read immediately or at least within 24 hours, but, when stored at 4° C in the dark with over 90% cell viability, trays may be readable up to 48 hours.

Calculations

No special calculations, as such, are necessary for the lymphocyte crossmatch procedures. Standard dilution techniques are used.

Results and Test Interpretation

Note: See ASHI Manual chapter entitled "Interpretation of Crossmatch Results."

1. An inverted fluorescence microscope is used to visualize the reaction that has occurred within each well on the HLA typing tray. Living lymphocytes are differentiated from dead cells by their color. Dead cells are a red color (stained with Ethidium Bromide). Live cells are green (stained with Acridine Orange).
2. The Crossmatch Tray Format worksheet should be completely filled out. This includes name of patient or donor, date of typing, initials of technologist performing test, type of crossmatch, temperature used for test, and cell viability.
3. Verify that the Crossmatch Tray Format worksheet and tray labeling match on each tray just prior to reading the tray.
4. Manually read trays in a serpentine fashion. (Read across row 1 [wells A-F], then back across row 2 [F-A], etc.)
5. Record results on the correct worksheet according to the following scale and Notes 1:

Score	Interpretation	% Dead Cells
1	Negative	0-10%
2	Doubtful Negative	11-20%
4	Weak Positive	21-50%
6	Positive	51-80%
8	Strongly Positive	81-100%
0	Not Readable	n/a

Note: Routinely, the negative control score is recorded exactly as it is interpreted, and all other scores are recorded with the background (i.e., negative control score) subtracted.

1) If the readings are equal to or less than the negative control, then the reader will record the reading with the background already subtracted off or indicate on the worksheet in another appropriate manner that the background may have been higher than normal.
2) All actual scores will be recorded if the reaction scores are above the negative control reading to aid in the interpretation of the crossmatch.

6. Interpret the results as follows (see ASHI Manual chapter entitled "Interpretation of Crossmatch Results"):
   a. Any positive reaction that is 11% above the negative control should be interpreted as a POSITIVE CROSSMATCH.
   b. A negative reaction that is equivalent to or less than 11% of the negative control should be interpreted as a NEGATIVE CROSSMATCH.
   c. If the controls do not react as expected, notify the laboratory Supervisor (also see Procedure Note #2):
      1) Negative control score should be "2"or less (i.e., cell viability greater than 80%).
      2) ALS control should be positive for T and B cells with a score of "4" or more.
      3) ABS control should be positive for B cells with a score of "4" or more and score negative for T cells.

Procedure Notes:

1. **Multiple Temperatures.** Running multiple temperatures during a crossmatch procedure allows verification of consistency of reactions and titers. With the exception of CYNAP reactions, true positive reactions should remain at least as strong but normally increase in strength and usually titer with in increase in temperature.
2. **Criteria for Repeat Testing.** The following situations should be reported to the Director who will make the final decision as to whether the test can be reported or needs further action (e.g., re-read tray, validate calculations, redraw, repeat test, confirm with outside laboratory, etc.):
   a. Poor cell viability demonstrated by a high percentage of dead cells in the negative control wells.
   b. Positive control wells fail to respond as predicted.
3. **Dilution Technique for Minimal Amounts of Serum.**
   The following technique using a Hamilton-type syringe is faster and less expensive to use than the standard serial dilutions made individually with a standard pipettor. Although the actual serum concentration with the two methods may vary slightly due to the Hamilton needle "dead space" volume, crossmatch results (i.e., positive or negative) do not appear to be affected. A Hamilton syringe that dispenses 1µl and has a total volume of 50µl, 80µl or 100µl can be used with the technique.
   a. Make aliquots of:
      1) 1.5 ml 2.5% FBS/RPMI (diluent) in 1.5 ml polyethylene tube and
      2) 50 µl each serum to be tested (or remove tubes from -80° C freezer if already stored as aliquot).
   b. To reduce likelihood of carryover of strongly positive controls, it is recommended to dilute and plate them last, i.e., always start with negative control and unknown sera first.
   c. After thoroughly rinsing the syringe with deionized water, rinse the syringe once with 2.5% FBS/RPMI.
   d. Use the syringe to mix the serum aliquot to be dispensed and diluted.
   e. Draw up volume of serum needed to dispense 1 µl per well for 1:1 (neat) and have 20 µl left for next dilution. Examples: 23 µl for three trays with one cell suspension being tested, 26 µl for three trays with two cell suspensions being tested.
   f. Dispense 1 µl serum in each appropriate well using the Crossmatch tray format worksheet for the test being performed.
   g. If the correct amount of serum was drawn up and dispensed, the syringe will have 20 µl left. Adjust to proper volume (draw or dispense) if needed.
   h. With a biohazard absorbent tissue or equivalent, wipe off the outside of the syringe needle being careful not to touch the needle tip and inadvertently siphon serum out of needle.
   i. Immediately make 1:2 dilution by drawing up 20 µl 2.5% FBS/RPMI in syringe to give total volume of 40 µl serum plus diluent.
   j. Mix serum and diluent by using 200 µl polyethylene tube for dispensing and drawing up of mixture 3-4 times. Be careful to minimize bubbles in sample and syringe during mixing.
   k. Draw up volume of serum needed to dispense 1µl per well for that dilution and have 20 µl left for next dilution.
   l. Dispense serum and make 1:4 dilution per Steps f-k. Repeat again for 1:8 dilution. Each cycle changes the dilution by a factor of 2, i.e., 1:1 becomes 1:2, 1:2 becomes 1:4, and 1:4 becomes 1:8.
   m. After the first serum has been diluted and dispensed for all concentrations to be tested, rinse the syringe with deionized water at least ten (10) times, i.e., draw up full syringe volume and expel the rinse water into a biohazard waste container ten times. Rinse the syringe once with 2.5% FBS/RPMI.
   n. Repeat the process (Steps d-m) with each serum until all have been plated on the tray in the correct Crossmatch worksheet pattern.
4. Reduce Serum Carryover. Whenever possible, add sera and cells in the direction of most negative sera to most positive sera (e.g. Negative control to patient (or donor) to ALS or ABS) and most dilute sera to most concentrated sera (e.g. 1:8 to neat) to reduce carryover. Wipe syringe needles or dispense drop when going from a higher concentration (1:1) to a lower one such as from one tray or row to the next or one serum to the next.

Limitations of Procedure

1. The cell concentrations must be proper to maximize the detection of antibody. In particular, cell suspensions that are too concentrated may not be able to detect weak antibodies in the serum.
2. The AHG concentration must be proper to maximize the detection of antibody. See antiglobulin QC procedure to determine the optimal AHG dilution to use.

References

1. *The American Association for Clinical Histocompatibility Manual*, 1981.
2. *American Society for Histocompatibility and Immunogenetics (ASHI) Laboratory Manual, 3rd Edition*, A Nikaein, ed., American Society for Histocompatibility and Immunogenetics, Lenexa, pp. I.B.1, I.C.1, I.C.2, 1994.
3. *NIAID Manual of Tissue Typing Techniques*, 1979.
4. Terasaki PI and McClellend JD. Microdroplet Assay of Human Serum Cytotoxins, Nature 204:998, 1964.

AHG Premixed with Complement: Streamlining for Protocols

Laura D. Roberts and Anne Fuller

Purpose

In order to increase the efficiency and accuracy of the antiglobulin complement dependent cytotoxicity (AHG-CDC) procedure, AHG can be premixed with the complement and then added directly to the Terasaki trays. This eliminates the critical AHG timing step that has contributed considerably to the technical variation evident in the AHG procedure. It has been reported[1] that AHG incubation longer than two minutes can actually produce false negative assay results. It also eliminates an incomplete flick of the last supernatant wash that can give false negative results due to excess dilution of AHG. The addition of AHG premixed with diluted complement can help streamline the AHG-CDC procedure, allowing an increased number of tests to be performed simultaneously.

This modification of the AHG-CDC procedure can be used for antibody testing using frozen cell trays, fresh or frozen local panels and crossmatching.

Specimen

1. Serum or re-calcified plasma.
2. Target lymphocytes isolated from peripheral blood, lymph nodes or spleen (>90% viable).

Reagents and Supplies

1. RPMI 1640 medium supplemented with HEPES.
2. Rabbit serum as source of complement.
3. Goat-antihuman kappa light chain (AHG).
4. Appropriate AHG controls in addition to positive and negative assay controls.
5. Plastic backed absorbent pad.
6. Gloves.
7. Complement cups.

Instrumentation/Special Equipment

1. 5 µl multi-channel repeating pipettor.
2. Pasteur pipets.
3. Pipettor adjusted to 200 µl.
4. Pipettor adjusted to 1000 µl.
5. 200 µl pipet tips.
6. 1000 µl pipet tips.
7. Centrifuge with rotor capable of holding trays and generating appropriate g forces.
8. Vortex.

Calibration

Calibrate centrifuge per manufacturer's instructions.

Quality Control

COMPLEMENT AND AHG SHOULD NEVER BE DILUTED AND REFROZEN. Make dilutions at the time of use.

Undiluted AHG should be stored at -70 to -80º C in small aliquots (5-10 µl) until needed. Depending on laboratory workload, 2-4 ml of AHG should be divided into 5-10 µl aliquots at a time. Bulk quantities of undiluted AHG can be stored in larger volumes indefinitely.

Initial characterization of AHG should include checkerboard titrations of well-characterized HLA antisera that demonstrate CYNAP reactivity. Normal human serum should be titrated as well, testing for any inherent toxicity found in the AHG mixture. Re-characterization of AHG should be performed each time a new bulk quantity is thawed to make aliquots or every 6 months (whichever comes first) to insure continued potency of AHG. The AHG quality control procedure can be found in the QUALITY CONTROLS Section of the ASHI manual.

Procedure

1. After initial incubation of cells and sera in the antibody screening or crossmatching procedure being used, wash Terasaki trays by adding 5 µl of RPMI to each well. To prevent carryover with the pipettor, click out between rows.
2. Centrifuge trays at 800x g for 10 seconds.
3. Flick trays to remove excess wash solution. Vortex trays to resuspend cells.
4. Repeat steps 1 – 3 three times.
5. Add 5 µl of AHG/complement mixture to each well of the Terasaki tray (see Calculations below).
6. Continue with complement incubation and addition of stain for the antibody screening or crossmatching procedure being used.

Calculations

Each laboratory should perform characterization of AHG to determine the optimal working dilution for AHG diluted in complement (see Procedure Note #1). Optimally, AHG is used with diluted complement. To obtain the appropriate concentration of AHG in diluted complement, base calculations on the following data from characterization of a current AHG lot commercially available:

---

1. For complement used at a final dilution of 1:1.5:
   a. To 5 µl aliquot of undiluted AHG, add 200 µl of RPMI.
   b. To 1 ml of undiluted complement, add 350 µl of RPMI.
   c. To the dilute complement, add 150 µl of dilute AHG.
   This is the formulation for a final concentration of AHG 1:400 in complement diluted to 1:1.5.

2. For complement used at a final dilution of 1:2:
   a. To 5 µl aliquot of undiluted AHG, add 200 µl of RPMI.
   b. To 1 ml of undiluted complement, add 800 µl of RPMI.
   c. To the dilute complement, add 200 µl of dilute AHG.
   This is the formulation for a final concentration of AHG 1:400 in complement diluted 1:2.

Results

Assay results should be reported based on the percentages of cell viability stated in the chapter titled "The Basic Lymphocyte Microcytotoxicity Tests" in this manual.

Procedure Notes*

*Modified from a Workshop handout by A. Fuller dated 10/98

1. **Determination of the Optimum Dilution of Complement and AHG.**
   As stated in the procedure section, optimally, AHG should be used with diluted complement, as undiluted complement is inhibitory to AHG augmentation. A dilution of AHG is chosen which is at least one dilution lower (less dilute) than one that will give maximal augmentation.
   a. AHG Titration.
      Titrate the AHG in checkerboard fashion with known CYNAP-reactive HLA alloantisera using a dilution of C′ (complement) that is routinely used in antibody screening and crossmatching procedures. Perform the standard AHG titration procedure using 0.001 ml AHG (serial dilutions of 1:25, 1:50, 1:100, 1:200, 1:400, 1:800, 1:1600) per well, incubate 2 minutes, then add C′. Include control wells with no AHG to establish that sera CYNAP. This titration will only tell you whether or not your source of AHG will actually augment cytotoxicity (not all available reagents do).
   b. AHG Plus Complement Titration.
      Titrate the C′ and AHG together as follows:
      1) Prepare five dilutions (undiluted, 1:1.5, 1:2, 1:4, 1:8) of C′ with RPMI 1640 as the diluent.
      2) Use each of the five dilutions of C′ as the "diluent" for the AHG, to prepare five sets of serial dilutions (1:100, 1:200, 1:400, 1:800, 1:1600) of AHG.
      3) Add 0.005 ml of each AHG/C′ dilution to rows of serially diluted sera which have been incubated with cells and washed 4 times. Use one series of AHG titrations in one dilution of complement per tray.
   c. Optimal Dilution.
      From these titrations determine the optimal dilution of AHG and C′ together that produce the highest titer of cytotoxic reactivity of each HLA alloantiserum. Normally, if the AHG concentration is too high in C′, reduced sensitivity of AHG-CDC is observed. Also, use of undiluted C′ dramatically reduces the sensitivity of AHG-CDC. From these data:
      1) Determine the volume of undiluted AHG to freeze in 1.5 ml bullet tubes. Normally this will be a small volume (0.005 ml) which can be diluted with buffer or RPMI in the bullet tube just prior to use.

2) Determine the optimum AHG dilution in C′. Dilute the AHG tenfold less than the optimum concentration in RPMI, i.e., if optimum concentration is 1:100, dilute the AHG 1:10.
3) Determine the C′ dilution that gives maximal CDC sensitivity.
4) Calculate the volume of diluent to add to the rabbit C′. The diluent is a mixture of diluted AHG (1/10 of total volume) and RPMI which is added to the C′ to obtain the final optimal dilution of AHG in C′.

d. Example: From titrations, AHG optimum was found to be 1:400 with the optimum dilution of in-house C′ being 1:1.5.
1) Aliquot undiluted AHG in 0.005 ml volumes in 1.5 ml bullet tubes. Freeze to -80ºC.
2) For use, thaw AHG, add 0.20 ml RPMI to make 1:40 dilution of AHG.
3) Thaw rabbit C′, measure out 1.0 ml C′ and add 0.35 ml RPMI diluent. Then add 0.15 ml of the 1:40 dilution of AHG (equals 1:10 dilution of AHG). Thus, the final volume of mixture equals 1.5 ml, where the final C′ dilution is 1:1.5 and final concentration of AHG is 1:400. For use, add 0.005 ml of AHG-C′ mixture per well of sensitized, washed cells.

2. AHG in high concentration can cause inhibition of complement activity. On the other hand, if the AHG concentration is too low, CYNAP antibodies will not react.

Limitations of Procedure

Lymphocyte antibodies other than HLA specific antibodies may produce positive results (cell death). A patient's antibody history, including sensitizing events and diagnosis, may be necessary to determine the nature of the reactivity.

References

1. Fuller TC, Fuller AA, Golden M, Rodey G. HLA alloantibodies and the mechanism of the antiglobulin-augmented lymphocytotoxicity procedure. Hum Immunol 56: 94-105, 1997.
2. Steen SI, Cheng CY, Ting A, et al. Simplification of the antiglobulin-augmented lymphocytotoxicity test: Addition of AHG to the complement. Hum Immunol 40 Supplement 1:136, 1994.

Premixing of C' and AHG for Standardization of AHG T Cell Crossmatches

Lori Dombrausky Osowski and Jeffrey McCormack

Purpose

The AHG (Anti Human Globulin) Crossmatch is the most sensitive lymphocytotoxicity method accepted for determining histocompatibility in the transplant recipient. The AHG crossmatch technique has always posed a technical challenge, which invites inconsistency for the technologist. Variable styles of "flicking," different wash protocols, the addition of a small amount of the carefully titered AHG, and critical timing of the addition of AHG and complement, all contribute to the variability and lack of standardization of AHG crossmatching . Premixing of the complement and AHG, along with long term storage of this reagent prior to using in the AHG assay eliminates much of this technical variation and allows for improved standardization of this assay between laboratories.

Initially it is useful to implement this technique of premixing AHG/Complement (AHG/C) using existing reagents that have already been quality controlled and validated in the laboratory. The proper dilution of AHG/C can be determined while comparing to the classical AHG technique of adding these reagents separately. Later, as one or the other reagent must be replaced in the laboratory, the existing complement or AHG can be premixed with the other reagent under evaluation, in order to be able to evaluate one reagent change at a time.

Specimen

Appropriate samples as defined by the laboratory's protocol for the AHG technique crossmatching.

Reagents and Supplies

Please refer to Chapter I.B.4. in this procedure manual: *AHG Premixed with Complement: Streamlining for Protocols.*

Instrumentation/Special Equipment

N/A

Calibration

N/A

Quality Control

Please refer to Chapter I.B.4. in this procedure manual: *AHG Premixed with Complement: Streamlining for Protocols.*

Procedure

Please refer to Chapter I.B.4.in this procedure manual: *AHG Premixed with Complement: Streamlining for Protocols.*

Procedure Notes

1. The non-AHG assay is used to compare as a baseline for detection of a CYNAP antibody by AHG.
2. It may be necessary to test additional dilutions of AHG/C, depending on the initial results.
3. Premixing of AHG/C at the same final dilution as the classical AHG technique often results in enhanced CYNAP reactivity.
4. The AHG/Complement mixture may be stable "long term" if properly diluted and stored. This may be demonstrated by additional time studies of the premixed reagent for acceptable performance after long term (-80° C) storage. In crossmatch QC and standardization proficiencies of five Texas laboratories, premixed AHG/C reagent stored at -80° C was stable for up to three months.
5. Please note that the long-term storage has only been tested in these studies using complement at neat (no dilution). Anytime that an antibody is frozen or thawed, there is risk of losing titer or sensitivity. A similar risk factor is encountered with complement, i.e., loss of complement binding activity. This is due to protein denaturation

during freezing and thawing. The general laboratory rule is that antisera and complement should not be stored frozen if it has been diluted with any type of aqueous solution. The protein-rich serum serves as a "protective environment" for the antibodies, and thus should not be diluted significantly and stored frozen. This would increase the risk of antibody (AHG) or complement breakdown during long term storage and freeze/thaw cycles.

Results

The pre-mix AHG/C procedure should yield equal, or in many cases increasing, sensitivity to the traditional technique. In addition, the new methodology should result in intralaboratory and interlaboratory standardization.

Limitations of Procedure

N/A

References

1. Dombrausky, L, Button E, Gobeli M, Hansen L. The AHG Microcytotoxicity Technique can be Performed with Premixed AHG/Complement for T Dynal Cell Crossmatches and PRAS, Human Immunology, Volume 44, Supplement, 1995. Abstract/poster presentation.
2. Fuller T, Monitoring HLA Alloimmunization: Analysis of HLA Alloantibodies in the Serum of Prospective Transplant Recipients, Clinics in Laboratory Medicine, p 551-571,September 1991, Glenn Rodey, Editor.
3. Johnson AH, Rossen RD, Butler WT. Detection of Alloantibodies Using a Sensitive Antiglobulin Microcytotoxicity Test, Tissue Antigens Volume 2:215-221,1972.
4. Lorentzen D. Quality Control of Reagents, ASHI Laboratory Manual, 2nd Edition, 1990. p 646.
5. Lorentzen D, and DeGoey S. Techniques for Reagent Quality Controls of Serology and Cellular Methods, ASHI Laboratory Manual, 3rd Edition. VI.4.1.
6. Tissue Typing Reference Manual, 2nd Edition, 1987. MacQueen, J.M., ed. Southeastern Organ Procurement Foundation, pp. 16-10 – 16-13.
7. Steen SI, Cheng CY, Ting A, Vayntrub T, Dunn S, and Grumet FC. Simplification of the Antiglobulin-Augmented Lymphocytotoxicity Test: Addition of AHG to the Complement, Human Immunology, Volume 40, supp. 1, Abstracts , 1994, p. 136.

T and B Lymphocyte Crossmatches Using Immunomagnetic Beads

Smita Vaidya and Todd Cooper

Purpose

The immunomagnetic beads (IM beads) crossmatch technique is an extension of the complement mediated cytotoxicity assay in which T and/or B lymphocytes are isolated by IM beads. Various types of T and/or B cell crossmatches can be performed by either positive or negative selection of lymphocytes. There are several advantages in using IM bead crossmatches over traditional methods of lymphocyte isolation. First, IM bead crossmatches are much faster. It takes half as much time to perform crossmatches when lymphocytes are isolated by IM beads. Second, these crossmatches are far more accurate largely due to isolation of highly pure lymphocyte populations. In addition, a combination of IM separation procedures with improved live/dead discriminating stains provides easer interpretation and more accurate analysis Using fluorescent dyes,dead cells fluoresce red by ethidium bromide and live cells fluoresce green by acridine orange (AO) or carboxyfluoroscein diacetate (CFDA). The red/green color difference is much easier to detect by human eye than the red/gray observed in conventional assays using eosin/formalin stain and fixative. The conventional method involving eosin/formalin dye/fixative does not work when lymphocytes are isolated by IM beads.

Specimen

Acceptable Specimens

1. Peripheral blood obtained in acid citrate dextrose (ACD)
2. Splenocytes in media
3. Lymph node lymphocytes in media

Unacceptable Specimens

1. Peripheral blood obtained in heparin
2. Specimens not properly labeled (see chapter titled “Guidelines for Specimen Collection, Storage and Transportation” in this manual)
3. Specimens transported in fixative

Reagents and Supplies

Preparation/storage instructions for reagents are provided in the chapter entitled “The Basic Lymphocyte Microcytotoxicity Tests.”

1. RPMI/heat-inactivated fetal bovine serum (RPMI/HIFBS) medium
2. Anti-lymphocyte serum (ALS)
3. Anti-B cell serum (ABS)
4. Normal human serum (NHS)
5. Complement
6. Trypan blue
7. Ethidium bromide (EB): *health hazard-potential carcinogen*
8. Acridine orange (AO) or carboxyfluoroscein diacetate (CFDA)
9. India ink, hemoglobin, or commercial quenching agent (e.g., Fluoroquench, One Lambda, Inc.)
10. Light mineral oil
11. Terasaki trays
12. Insta-Seal cover slides (disposable, One Lambda, Inc)

Instrumentation/Special Equipment

Fluorescent microscope.

Calibration

Not applicable.

Quality Control

1. To insure the quality of immunomagnetic beads, lymphocyte preparations from two different donors should be isolated using the new lot of beads. The old lot should be tested in parallel with the new lot.
2. Complement, anti-human globulin, and control sera should be QC'd as described in the Quality Control section of this manual.
3. Cell/bead preparations should be crossmatched using the laboratory's standard techniques with the following controls:
   a. ALS
   b. NHS
   c. RPMI
   d. ABS
4. Inadequate cell isolation is suggested by the following scores in the ABS wells:
   a. >10% using a T lymphocyte preparation
   b. <80% using a B lymphocyte preparation
5. Cytotoxicity caused by beads or complement is suggested by a background score above 10% in the following wells:
   a. RPMI
   b. NHS
6. Reagent lots deemed inadequate should be returned to the vendor.

Procedures

Preparation of Crossmatch Trays

1. Prepare the list of patients' sera to be tested.
2. Fill out appropriate crossmatch tray sheets with patients' names, dates of each serum to be tested, potential donor's name, ABO typings of the donor and the patient, date of the crossmatch test, etc.
3. Dispense 2-5 µl of light mineral oil per well in each of the crossmatch trays.
4. Plate patients' sera with appropriate dilutions (per individual laboratory policy) along with ALS and ABS as the positive controls and NHS as the negative control.
5. Always rinse all Hamilton Syringes at least four times with deionized water before and after use. Also wipe the tips of the syringes while moving from serum to serum to minimize carry over.

Lymphocyte Preparation and Tray Plating

1. Using the methods for lymphocyte isolation by IM beads, described in chapter "Immunomagnetic Isolation of Lymphocyte Subsets Using Monoclonal Antibody-Coated Beads" in this manual, isolate appropriate subpopulation of lymphocytes by either positive or negative selection.
2. Adjust the concentration of lymphocytes to approximately 2-3 x 10^6/ml.
3. Label each tray with patient's name.
4. Plate 1 µl of viable and adjusted lymphocytes (approximately 2500 cells) per well using a Hamilton Syringe. Make sure that sera and cells are mixed.
5. Incubation time varies for each cell and crossmatch type. Determine appropriate conditions for each crossmatch using Table 1.
6. Add 5 µl of complement EB/AO (or CFDA) solution per well using a Hamilton Syringe.
7. Incubate at room temperature (RT) for the time specified in Table 1.
8. Add 5 µl of 10% hemoglobin solution (or 2.5% India ink solution or equivalent solution) to each well. These solutions quench the background, making reading easier. Carefully apply a 2" x 3" Insta Seal cover slide to each tray if covers are a standard practice in the laboratory.

Table 1. Conditions for IM bead crossmatches

Crossmatches	IM Bead	Pos/Neg Lymphocyte Selection	Incubation Condition			
			Serum		Complement	
			Time (min)	Temp (%C)	Time (min)	Temp (%C)
1. T cell NIH	$CD8^+$ or $CD2^+$	Pos. T	20	20-22	35	20-22
2. T cell extended	$CD8^+$ or $CD2^+$	Pos. T	45	20-22	90	20-22
3. T cell extended† one wash	$CD8^+$ or $CD2^+$	Pos. T	45	20-22	90	20-22
4. T cell AHG†	$CD8^+$ or $CD2^+$	Pos. T	30	20-22	60	20-22
5. T cell DTT‡	$CD8^+$ or $CD2^+$	Pos. T	adjust per crossmatch type			
6. B cell	$CD19^+$ or DR^+	Pos. B	45	20-22	90	20-22
7. B cold	$CD19^+$ or DR^+	Pos. B	45	0-4	90	20-22
8. B cell DTT‡	$CD2^+$	Neg. B	adjust per crossmatch type			

† For any of the above procedures, wash steps may be added as appropriate.

‡ For DTT crossmatches adjust incubation times and temperatures on the basis of crossmatch type.

Calculations

Not applicable.

Results

Read immediately. Although it is possible to read trays 24 hrs after staining, the cell viability decreases causing background death to increase. Grade the crossmatch reactions using the scoring method recommended by the ASHI Standards.

Procedure Notes

1. Some laboratories use parallel crossmatch trays run at different temperature combinations, e.g.:
   a. T cell XM = 4° C, 22° C and 37° C
   b. B cell XM = 22° C and 37° C
   These XM setups are used to gain reaction consistency and temperature titering data as well as to provide backups for the other trays in the set.
2. **TROUBLESHOOTING**
   a. If the blood sample is from a cadaver donor or 3-4 days old, isolate 12-15 x 10^6 lymphocytes from buffy coat using ficoll. Resuspend lymphocyte pellet in 5 ml **cold PBS** (0-4° C), add appropriate beads and follow steps 7-10 of the procedure.
   b. If the donor has a viral infection, do not use $CD8^+$ beads because $CD8^+$ beads will isolate virally activated T lymphocytes. Activated T cells are difficult to type as well as crossmatch. Under these conditions, use either $CD2^+$ beads, or perform negative selection of T cells.
   c. If cell preparation from peripheral blood or spleen contains excess monocytes or granulocytes, remove by adding iron particles.
   d. Occasionally high background death results from toxicity of India ink. Check India ink for bacterial contamination and dilute India ink further. Hemoglobin works best as a quenching agent.

Limitations of Procedure

1. Use of AHG with B-cell targets may lead to false positive results due to AHG binding with surface immunoglobulins. Although the authors do not use AHG/B cell techniques, some laboratories do have such protocols.
2. A serological crossmatch using non-fluorescent dyes does not work when the lymphocyte isolation is performed with IM beads.
3. Since the IM bead crossmatch technique requires fluorescent dyes, the completed trays must be protected from ambient light sources to retain maximum fluorescent potential.

References

1. Povlsen JV, Madsen M, Rasmussen A, Strate M, Graugaard BH, Birkeland SA, Hansen HE, Fjeldborg O and Lamm LV: Clinical applicability of the immunomagnetic beads technique for serologic crossmatching in renal transplantation. Tissue Antigen 38(3):111, 1991.
2. Johnson AH, Rossen RD and Butler WT, Detection of alloantibodies using a sensitive antiglobulin microcytotoxicity test. Tissue Antigen 2:215, 1972.
3. Vaidya S, Orchard P, Schroeder N, Haneke R, Brooks K, Thomas A, Corba A, Asfour A, and Fish JC, Clinical importance of premortem blood lymphocytes in cadaver donor tissue typing. Clinical Transplantation 9: 165, 1995.

Interpretation of Crossmatch Results

Diane J. Pidwell

Purpose

This chapter will review some of the factors to consider when interpreting the various crossmatch procedures used in solid organ transplantation.

Procedures

Refer to chapters in this manual for: cell isolation; complement dependent cytotoxicity protocols and flow cytometric protocols for crossmatching and antibody identification, blocking techniques, absorption procedures, and ELISA protocols for antibody identification.

Introduction

Serologic crossmatches, in which serum from transplant candidates is incubated with donor cells and reactivity is detected by cell lysis or flow cytometry, are performed to detect the presence of pre-formed anti-donor specific antibodies present in the serum of potential recipients. The cytotoxic crossmatch was first employed in the histocompatibility laboratory in the early 1960's in renal transplantation as a means of identifying donor-recipient combinations that were at risk for hyperacute rejection, the explosive coagulopathy mediated by antibody and complement that occurs in the immediate post transplant period and inevitably ends in graft failure.[51,104,107] At that time hyperacute rejection of allografts was a major obstacle to successful transplantation and the cytotoxic crossmatch proved to be a highly effective means of detecting, and thereby avoiding, donor-recipient combinations at risk for hyperacute graft loss.[74]

However, the science and practice of transplantation have changed dramatically in the last thirty-plus years. Experience has made it painfully clear that hyperacute rejection is not the only immunological threat to allografts but that acute and chronic rejection represent formidable obstacles to graft function and survival as well. Additionally, the pioneering efforts in renal transplantation have now blossomed into the routine transplant of a wide variety of solid organs and tissues. Heart, liver, lung, small bowel, pancreas, bone marrow and stem cell transplants are now commonplace and the diversification will obviously continue as novel procedures for transplantation of bones, joints, nerves, skeletal muscle, retinal tissue, and composite tissues such as the hand or larynx become more widely applied. Much of this expansion has been made possible through the availability of new pharmaceutical agents and immunosuppressive regimens that can effectively prevent or reverse the majority of acute rejection episodes. Here again the trend promises to continue as agents are sought that can more effectively prevent humoral sensitization, or the vascular sclerosis and fibrosis of chronic rejection. Now that methods for xenotransplantation and induction of donor-specific tolerance have appeared on the horizon, transplantation may soon become as commonplace as open heart surgery. Laboratory practices have, of course, evolved in parallel with these changes. More sensitive crossmatch techniques have been developed along with new methods of detecting and characterizing alloantibodies. With the advent of DNA typing and myriad new MHC alleles, new methods for HLA matching for solid organ transplant are being developed.

In this rapidly evolving field, it is difficult to evaluate the impact of one innovation before being overtaken by the next. Currently, it can prove challenging to determine the relevance of DNA typing, ELISA antibody screening, and a positive B cell flow crossmatch when evaluating a candidate for renal transplantation let alone for a small bowel or hand transplantation. In a field evolving at this pace the relevance of the different donor specific crossmatches is not always clear and we find ourselves searching for assays that can help us assess the risk of cellular rejection, as well as humoral rejection, and for means of identifying grafts that are at risk of developing chronic rejection. At this juncture it is critically important that we scientifically evaluate the implications for graft survival of each technique employed in our laboratories, and determine if crossmatch results are only useful for telling us which transplants to avoid or if these assays can be used to identify patients at risk for acute rejection, aid in the selection of immunosuppressive regimens, and help in the medical management of transplant recipients. It is no longer enough merely to avoid hyperacute rejection, the challenge now is to prolong graft survival not just for years, but for decades.

What information can a crossmatch provide? Ideally, a serologic crossmatch should specifically and sensitively detect the presence of pre-formed antibodies that can bind to transplanted donor tissue and cause immediate and/or irreversible damage to that tissue. The less-than-ideal reality is that there is no single crossmatch procedure currently available which can unequivocally detect all of the antibodies capable of causing graft injury or rejection and at the same time detect only antibodies that forebode graft injury and eventual graft loss. The interpretation of crossmatch results therefore now involves integrating an ever increasing amount of information in an attempt to approximate the risk of antibody and cell-mediated injury to the graft in any given transplant. Interpreting a crossmatch today will perforce include consideration

of factors such as: 1) What antibodies represent a threat to graft function and survival? 2) Has the patient experienced sensitizing events such as pregnancy, transfusion, or a prior transplant? 3) Has antibody ever been detected in this patient previously? If so, when was it detected, what techniques were used to detect it, and what was the specificity of the antibody? 4) What target antigen was used to detect the antibody? 5) What is the immunoglobulin isotype of the antibody? 6) Is there auto-antibody present? 7) What organ is to be transplanted? and 8) What crossmatch techniques were used?

Additionally, it is likely that the pre-transplant crossmatch will not be the last assay the physician requests from the histocompatibility laboratory. With increasing frequency, donor specific crossmatches and antibody screens, are being employed post transplant to monitor the development of donor-specific responses. Hence, this chapter will also consider the implications of a positive post transplant crossmatch.

The list of considerations given above is certainly not all inclusive. However, this chapter will be limited to a review of the literature addressing those eight considerations and to the interpretation of post transplant crossmatches. The reader is strongly encouraged to visit the literature as a means of accessing the experience of other histocompatibility laboratories and researchers and as a means of keeping abreast of the most current information available in this highly dynamic field.

Some Factors to Consider when Interpreting Crossmatch Results:

1. What antibodies represent a threat to graft function and survival.

The reason for performing a donor specific crossmatch either pre or post transplant is to alert the physician to the presence of donor specific antibodies that present a risk of causing graft injury and graft loss. A major antibody-mediated threat to graft survival is hyperacute rejection,[74,88,107] and the preponderance of evidence indicates that in ABO compatible grafts[107] the most common cause of hyperacute rejection is anti-MHC class I specific antibodies.[20,21,46,48,103] There have been instances reported where hyperacute rejection has been caused by anti-MHC class II antibodies,[68,86] anti endothelial cell specific antibodies,[8,19,56,88] and by tissue specific antibodies,[80,89,105] however those reports are more limited in number and usually are seen in replantation patients and parous females. In general, the antibodies that represent the major risk factor for hyperacute rejection are anti-MHC class I specific antibodies.[28] However, if we consider all forms of rejection, not just hyperacute rejection, then the most dangerous antibodies include anti-MHC class II-specific as well as anti-MHC class I-specific antibodies.[43,82]

Unfortunately, the anti-MHC antibodies that represent the greatest threat to graft survival, are also the antibodies most commonly seen in transplant candidates,[10] and the detection of donor reactive anti-MHC antibody in a crossmatch is the single most important immunologic contraindication to transplantation.[73] It is crucial therefore, to define the antigenic specificity of any circulating antibody encountered in transplant candidates. That does not mean that the MHC allelic specificity is an absolute requirement, but that anti-MHC reactivity must be recognized.[46,110]

Until quite recently it had often been difficult to prove the anti-MHC specificity of many antibodies encountered in transplant candidates because definition of specificity required time-consuming dilutions, absorptions, repeated analysis, and occasionally even blocking studies.[20,21,45,46] The recent availability of solid phase antibody characterization systems, which use reasonably pure preparations of MHC molecules as the target antigen, has made this task somewhat easier.[13,43,48,67,70,82,110] Reactivity in solid phase assays clearly helps to define the MHC class I and class II specificity of an antibody. Use of the information gained from these more definitive assays will aid in the interpreting crossmatches, which continue to require whole cells as target antigen and are therefore subject to "false positive" results caused by non-MHC directed antibodies.[12]

The sensitivity of antibody screening protocols, i.e., the ability to detect low titer antibody, has been another problem area in efforts to detect and characterize alloantibody because complement dependent cytotoxicity(CDC) screening protocols have been shown to be much less sensitive than the widely used flow crossmatch protocols.[37,73] With this disparity in sensitivity, it has become fairly common to see patients with a negative antibody screen (PRA) by CDC with a positive flow crossmatch. Encountering a donor-reactive antibody for the first time in a final crossmatch means that the antibody specificity is unknown and interpretation of results is difficult. Without knowing the antibody specificity, estimations of risk must assume the worst case scenario, that the antibody is anti-MHC specific, and transplants can be unnecessarily postponed or denied. The new solid phase assays, as well as cell-based flow antibody screening protocols,[48,90] have aided in this regard, as well, because the sensitivity of both the flow and the ELISA screens has been shown to exceed the sensitivity of CDC assays and to approach or equal the sensitivity of the flow crossmatch.[48,70,90,110]

2. Has the patient experienced sensitizing events such as pregnancy, transfusion, or a prior transplant?

Anti-MHC specific antibodies are usually elicited by exposure to alloantigen through pregnancy, transfusions, or prior transplants.[84] It would logically follow then, that if a transplant candidate has never been pregnant, transfused, or previously transplanted, alloantibodies should not be present. Unfortunately, lack of any known sensitizing event cannot safely be interpreted to mean lack of sensitization. In the histocompatibility laboratory it is never safe to conclude that the absence of documentation of an event is in any way equivalent to the absence of the occurrence of that event. First and foremost, the histocompatibility laboratory can never be sure they have been informed of every potentially sensitizing event. Secondly, it is possible that neither the patient nor the physician are aware of a sensitizing event, for example a woman may experience a miscarriage before she even knows that she is pregnant, or blood transfusions could have been administered without the transplant team or the patient being clearly informed. Thirdly, there have been reports of natural anti-MHC antibodies and of anti-microbial antibodies that can cross react with MHC antigens.[46,47] Therefore, even in

the absence of any known sensitizing event, all transplant candidates should be screened for preformed antibody and it should be assumed that any antibody detected in a transplant candidate represents a risk for graft injury until laboratory results can clearly demonstrate otherwise.

3. Has antibody ever been detected in this candidate previously? When was it detected, what techniques were used to detect it, and what was the antibody specificity?

Clearly, the presence of pre-formed anti-MHC antibody in a transplant candidate can represent a significant risk for graft injury. The evidence is indisputable: hyperacute rejection is an antibody and complement mediated event that is frequently initiated by anti-MHC antibodies. This threat of immediate and irreversible graft loss as well as the fact that antibody can also be involved in acute and chronic rejection,[2, 48, 56, 106] makes it crucial to recognize the presence of alloantibody and to determine the MHC reactivity of that antibody.[82, 43] A complete antibody history and thorough characterization of antibodies pretransplant can be crucial for the successful post transplant management of graft recipients. In renal and cardiac transplant candidates a pretransplant PRA of >10% has been shown to identify a group of patients who are at higher risk for post transplant complications such as primary non function, acute rejection episodes, and graft loss.[27,52,92,102] Even patients who have negative T and B cell CDC crossmatches and low PRAs at the time of transplant are at increased risk for graft injury and graft loss if their pretransplant PRA was ever>10%. Early recognition of these immunologically reactive patients allows transplant physicians to tailor the clinical pathway such that the patients at highest risk for rejection can be more closely monitored post transplant and increased immunosuppressive therapy can be initiated early when rejection is encountered.

As mentioned above, different antibody screening techniques vary in their sensitivity and in their specificity for MHC antigen. ASHI standards continue to require CDC screening, which is informative because the CDC assay clearly demonstrates that the alloantibody is capable of complement activation and therefore of mediating graft injury. But, CDC assays alone are often inadequate because of their low sensitivity and because they require whole cells as targets which makes them susceptible to "false" positive results produced by non-MHC directed antibodies.[46] We have already discussed the difficulty with using an antibody screening protocol that is less sensitive than the final crossmatch because previously undetected antibody is first recognized at a point when time constraints prohibit thorough antibody characterization. Fortunately, those shortcomings can now be addressed through the use of the flow and ELISA screening protocols. The use of these more sensitive and specific screens should aid greatly in the interpretation of crossmatch results.

Using a crossmatch, whether it be flow or CDC, to screen before transplant is adequate as long as alloantibody remains detectable in the patient's serum. But how does the laboratory quantify the risk for a candidate that has a history of MHC specific antibody but whose antibody is no longer detectable in their circulation? Or, what is the risk of graft rejection in a patient who has a current negative crossmatch, and no history of alloantibody, but who has a positive crossmatch with historic serum? To immunologists who have been raised on the tenet that specific immunity is defined as having memory, the answer is obvious; if the antibody was there once it will reappear upon re-exposure to the antigen. However, "for every rule there is an exception" and in solid organ transplantation this appears to be one of those exceptions.

A number of cases have been reported where patients have been transplanted across past positive, current negative crossmatches and their graft function and short term graft survival has been equivalent to that of grafts in recipients with both historic and current negative cross matches.[16,17,20,50 however see 21] These reports suggests that anamnestic responses may be absent, or at least diminished, in some previously sensitized solid organ recipients. Of course, there are also instances where antibody production does recur rapidly post transplant, but even in those cases graft loss is not universal.[47,87] In some instances, if antibody is detected early and if it can be reduced by plasmapheresis,[2,3,18,49,62,78] IVIg administration[30,32,39,65] or in some cases just by altering the immunosuppresion regimen[108] graft function can be restored and maintained. It is also possible that alloantibody may reappear slowly. Since high antibody titer is crucial for hyperacute rejection these grafts are not subject to immediate loss but appear to be at increased risk for acute and chronic rejection (see the section on post transplant crossmatching below).

It is unclear why antibodies recur in some patients and not in others, or why rescue efforts are successful only part of the time. It is also not clear if long term graft survival is compromised in past positive/current negative recipients since the reports did not include long term follow up. Transplant researchers and laboratory personnel are constantly searching for new assays that can clearly delineate a patient at risk for an immunological response from patients who are not at risk and the best assays available at this time are still alloantibody screens and crossmatches. When using a final crossmatch as a measure of immunologic risk to a graft, a positive crossmatch with historic serum is an indicator of some risk but that risk is apparently not, by itself, sufficient to justify denying the patient a transplant.[16]

It should be mentioned that there is a problem with relying on published reports when estimating the amount of risk implied by a positive donor specific crossmatch. That problem is that the risk is probably not the same at every transplant center. Several recent multi-center studies have shown that one factor that can significantly influence transplant outcome is the "center effect."[22,35,70,84] Evidently, medical practices differ at different transplant centers and what may be a sign of high risk at one center may not carry the same weight at another center

Unfortunately, many centers have too few patients in each category, or have insufficient follow up data on their patients to produce clear conclusions for their own center. In some centers the histocompatibility laboratory will receive no post transplant information on patients until they reappear on the list as replant candidates. Interpreting crossmatches can be a tricky proposition and it is best done when the people interpreting the results have an complete, scientifically derived, concept of center specific outcomes. In the absence of sufficient center specific data however, risk estimation is forced to rely upon published reports.

One final note on factoring historic antibody characterization information into interpretation of crossmatch results. There are a group of transplant candidates who can be characterized as immunologically hyper-responsive and who appear to be at higher risk than other sensitized patients. These are the patients who are highly sensitized after a minimal immunization. Consider patients sensitized by blood transfusions. Scornik et.al.[84] have suggested that it can take as many as seventy units of blood to stimulate anti-MHC class I antibody, whereas Cicciarelli[25] states that five or more units will suffice to establish the responder pattern. Despite these estimates, it is clear that some patients who have received as few as one or two units of blood develop broadly reactive antibody that persists for years. It may be beneficial to view these patients as being at higher risk for immunologic responses of all kinds and to regard them as high risk patients that should be closely monitored post transplant.

4. What was the antigen source used for antibody identification and crossmatching; T cells, B cells, monocytes, endothelial cells or isolated HLA antigen?

Until novel procedures become available for extracting and purifying MHC antigens from donor cells, which will permit the use of MHC-specific solid phase testing for crossmatching, histocompatibility laboratories must continue to rely on whole cells as donor specific targets for crossmatches. Lymphocytes are a readily accessible source of donor MHC antigens but the disadvantage of using lymphocytes is that they express antigens not normally found on transplanted organs, they lack tissue-specific antigens, and they are frequently targets for autoantibodies. This complexity of antigen expression and reactivity makes interpretation of cell based assays difficult.

T lymphocytes normally express only MHC class I antigens but when human T cells are activated they will express class II antigens as well. This is a fact worth remembering when working with donor cells where treatment or sepsis may have triggered lymphocyte activation. Human B cells express both MHC class I and class II antigens under normal conditions. Interestingly, B cells usually express more MHC class I molecules per cell than T cells and therefore can be more sensitive to complement dependent lysis in instances where anti-class I antibodies are present in low concentrations.[12,14,45,75] B cells also have a propensity for binding autoantibodies.

Since anti-MHC antibodies present the greatest threat to graft survival in primary as well as replant recipients, assays designed to detect and characterize antibodies need to be optimized to detect anti-MHC reactivity. Using protocols that depend on separated T and B lymphocyte populations is one method of optimizing the information gained from the results. When interpreting assays that use separated lymphocytes as targets, the presence of both T and B cell reactivity implies anti-MHC class I specific antibody. T cell reactivity in the absence of B cell reactivity is probably the result of non-MHC class I specific antibodies since both cell types express class I antigens. This pattern of reactivity can be misleading however, and further characterization of the specificity of these antibodies is necessary before dismissing them as irrelevant. B cell reactivity, in the absence of T cell reactivity is more complex to interpret. This pattern of reactivity indicates either weak anti-class I antibody, *and/or* anti class II antibody *and/or* autoantibody.[12,14,45,46] Obviously, B cell reactivity is much more difficult to characterize and identification of anti B cell reactivity has traditionally required absorptions with platelets or cells for resolution. Luckily, conditions have improved and again flow and ELISA techniques can help. If B cell reactivity is due to weak anti-class I antibody, a flow screen that uses class I coated beads or T lymphocytes as targets should also be positive because flow cytometry is capable of detecting the lower titers of antibody that can cause positive B cell reactions but are too weak to be picked up on T cell CDC assays. The absence of reactivity to T cells or to class I coated beads in flow analysis indicates two possibilities, either the antibody which is reacting with B cells is not MHC class I directed, or it is not IgG. This latter conclusion can be drawn because most flow assays employ IgG-specific secondary antibodies. In either case, that antibody has a low risk of producing hyperacute rejection in primary transplant candidates, and a positive crossmatch caused by that antibody is not sufficient reason to deny transplantation in those candidates. In candidates awaiting retransplantation or in high risk patients however that antibody is of more concern.[3] In those patients, further antibody characterization is necessary and it may be helpful if that evaluation includes the use of MHC class II coated flow beads, or ELISA systems that contain class II antigens.[82] If autoantibody is suspected it should be confirmed with auto crossmatches and auto absorption, and the absorbed serum should be retested to clearly demonstrate that no previously obscured anti-MHC antibodies are present.[12] Happily, patients that require that level of investigation are rare and since B cell reactive antibodies are of concern primarily in replant and highly sensitized candidates, these investigations may not be worth pursuing except in that subpopulation of patients. In any case, putting in the effort to thoroughly characterize antibodies during preliminary screening has its benefits later during the interpretation of final crossmatch results.

Human monocytes also express both MHC class I and class II antigens and additionally express antigens that are shared with endothelial cells. Use of monocytes in antibody screening and crossmatches can therefore permit detection of anti-endothelial cell antibodies that have been implicated in causing hyperacute rejection.[80] The ability to detect these antibodies has led some laboratories to perform monocyte based assays. However, monocytes tend to bind antibody through non-antigen specific mechanisms e.g., through F_c receptors, which makes interpretation of monocyte based assays difficult. Additionally, when one considers that reports of graft loss due to anti-monocyte or anti-endothelial antibodies have been sparse, the routine use of these assays is difficult to justify. However, each center must decide for itself if ignoring anti-monocyte or -endothelial cell antibodies represents an acceptable risk.

As mentioned earlier, there is evidence that solid organ grafts can express antigens that are not present on lymphocytes and it has been suggested that antibodies which bind to those antigens may cause hyperacute graft loss.[55,80,89,105] This could be interpreted to mean that tissue specific crossmatches should be performed for solid organ transplantation especially in replant candidates and multiparous females since both of these groups have previously been exposed to allo-

geneic tissue and tissue specific antigens. But since definition of these antibodies depends on the availability of organ-specific cells and since cells from donor organs are unlikely to ever be rapidly available for cadaveric crossmatches, the rare hyperacute rejection due to tissue specific antibodies will probably remain a phenomenon that can only be diagnosed after the fact. One can only hope that the relevant tissue-specific and endothelial cell antigens will soon be identified and isolated so they can be incorporated in solid phase assay systems.

Since occasionally cases continue to be reported where hyperacute rejection is diagnosed in the absence of detectable anti-MHC antibodies[61] it is prudent to remember that any transplant, even those with negative CDC and flow crossmatches, represents a risk, and that no crossmatch, no matter how sensitive, guarantees the absence of antibody mediated graft injury.

5. What is the isotype of the antibody? and 6. Are auto antibodies present?

IgM antibody are frequently quoted as presenting minimal risk for solid organ graft injury or hyperacute rejection.[19,21,50,79,96 see however 46] (A fact that has always eluded me to some extent because natural anti-ABO antibodies are IgM and they damage solid organ grafts quite readily.[34]) Regardless, there is an abundance of evidence in the literature to support the opinion that it is safe to transplant if the crossmatch is negative in the presence of an IgM reducing agent such as DTT or DTE.[4,9,91,97] The crux of this argument appears to be that the majority of IgM antibodies are autoantibodies, and autoantibodies are not detrimental to graft survival.[10,20,36] Several authors have in fact suggested that autoantibody may actually be beneficial for graft survival.[27,93,100] A cautionary word should be inserted at this point however. Firstly, there is published evidence that anti-MHC specific antibodies can be IgM and that IgM anti-MHC antibodies can cause rejection of solid organs particularly in replant recipients.[12,20,110 see however 46,79] Secondly, IgA is also reduced by DTT or DTE treatment and graft damage due to IgA has been reported.[27,108] This makes it unwise to assume that all antibody activity reduced by DTT presents no threat to organ survival. Thirdly, autoantibodies can be IgG and as long as the autoantibody is not obscuring anti-MHC specific IgG antibodies there is apparently minimal risk in proceeding with the transplant.[12] Finally, with the current state of technology IgM is more accurately identified through the use of IgM-specific antisera in flow or ELISA assays.[7,84] As William Braun commented in his paper on managing highly sensitized patients:[12] DTT reduction is circumstantial evidence of autoantibody, and the true test of autoantibody, no matter what the isotype, is that it is removed by absorption with autologous cells. The important point is, IgM antibodies are not always innocuous antibodies and transplanting in the presence of cytolytic anti MHC antibodies, whether they are IgG, IgA, or IgM, carries increased risk.

6. What organ(s) or tissue(s) is to be transplanted?

It is not absolutely necessary to perform a prospective crossmatch for all solid organ transplants since the stringency for requiring a negative crossmatch is different with different organs. It makes sense then to determine the requirements for each organ at your center so as to limit the preservation time for organs while maintaining good patient care.

For renal transplants the historical evidence and the ASHI standards are clear, negative prospective donor specific crossmatches are required.[5,51,74,107] These cross matches must include a sensitive CDC and/or flow T cell cross match, and it is recommended that they include a B cell crossmatch as well. Pancreas and renal/pancreas transplants are subject to significant post-transplant morbidity which adds inherent risk to the procedure. Since it is also difficult to biopsy pancreatic grafts to diagnose acute rejection it can be argued that pancreas transplantation should be pursued only under the most favorable immunologic conditions which can be defined as including a negative T cell flow and/or a negative B cell CDC crossmatch (see explanation of the relationship of antibody titer and cellular reactivity in #8 below).

For liver transplants there is a significant amount of data to support the opinion that a negative crossmatch is not a prerequisite for successful transplantation or for long term graft survival.[34,60,94 see however 71] There have been reports of hyperacute rejection of liver grafts[44] but those situations have evidently been rare and the majority of published evidence indicates that preformed donor-specific alloantibody does not jeopardize the function or the post transplant survival of liver allografts. Each transplant center must decide for itself if a liver transplant would be canceled or if the immunosuppressive therapy would be handled differently because of a positive T cell CDC crossmatch. If the transplant would proceed and treatment would not be changed in light of a positive crossmatch it would seem acceptable to perform either a retrospective crossmatch or no crossmatch at all for liver transplantation. Although this decision may seem at odds with the ASHI standards for non-renal organ transplantation which states that high risk patients should have prospective crossmatches,6 it is possible that there is really no such thing as an immunologically high risk patient in liver transplantation. In a time of capitated contracts and hospital cutbacks, laboratories may be required to eliminate tests that do not directly impact clinical pathway decisions.

It is slightly more difficult to determine the importance of a positive prospective crossmatch in situations where another organ is to be transplanted in combination with the liver. There is evidence to support the conclusion that the liver will "protect" the additional organ(s) so that a positive pretransplant crossmatch may not be a contraindication for these transplants.[67] But in cases of multi-organ transplants the results of a prospective crossmatch can influence decisions concerning which organ to transplant and perfuse first and in some instances it may be deemed too risky to transplant a patient across a positive crossmatch that is due to anti MHC-class I or class II antibody. Therefore, in multi organ transplants, even those including a liver, a prospective crossmatch should be performed whenever possible but certainly on high-risk patients as defined in the ASHI standards.[6] In the final analysis the physicians and the laboratory director may have to decide when it is and when it is not "possible" to prospectively crossmatch non-renal transplant candidates.

In lung, heart, or heart lung transplantation preservation time for the organ is a major concern since prolonged ischemia time can by itself cause irreversible organ damage.[29,42,56] One way to limit preservation time is to remove the prerequisite for prospective crossmatching which allows surgeon to begin the transplant prior to the reporting of results. However, since hyperacute rejection has been described in heart and lung transplants[33,52,53,56] a positive T cell CDC crossmatch is a contraindication for transplantation.[36] The solution for this dilemma is to carefully screen all heart and lung transplant candidates prior to listing and periodically throughout their time on the waiting list. This allows patients with anti-MHC class I antibody to be ear-marked as requiring a prospective crossmatch. In the absence of pre-formed alloantibody, a concurrent or retrospective crossmatch will usually suffice. For this system to function effectively the antibody screening techniques must be *as* sensitive or *more* sensitive than the final crossmatch procedure, and the physician and the laboratory *must* be informed of all potentially sensitizing events. As stated in section two above, this approach does carry extra risk because of unidentified or unreported sensitizing events. In heart and lung transplantation however this extra risk may be offset by the advantages of reducing cold ischemia time. In each situation discussed in this chapter there seems to be at least one exception and with deferring prospective crossmatches for heart transplantation that exception is patients where a ventricular assist device (VAD) is being used as a bridge to transplant. VAD recipients can receive numerous units of blood and blood products during VAD placement and often develop alloantibody before an appropriate donor can be found. Because of the frequency of alloantibody production in VAD patients many institutions now use immunosuppressive therapy post transfusion in an attempt to prevent allosensitization. Unfortunately, current immunosuppressive options are relatively ineffective at preventing B cell activation and these therapies may simply delay alloantibody production, sometimes for months after sensitization. Until better regimens for preventing B cell activation become available VAD patients should be treated as high risk patients and should either be followed very closely for antibody development using a screening method that is at least as sensitive as the crossmatch which will be used to rule out transplantation or they should require prospective crossmatches.

Heart and lung transplant, are frequently areas that continue to require B cell crossmatches despite the fact that the literature is not entirely clear as to the relevance of the results.[15,27,52] Since it has been demonstrated that cardiac and pulmonary dysfunction is more commonly seen in patients with panel reactive antibody greater than 10% and positive flow crossmatches,[7,18,66] a case can be made for performing heart and lung transplants only under the most immunologically favorable conditions such as a negative B cell CDC and/or T cell flow crossmatch. This again is one domain where each institution should define their clinical pathway based on their own data on graft survival and patient outcome.

Because of limited application, and my limited experience with other allotransplants such as small bowel, larynx, brain cells, nerves, muscle, joints, composite tissues, and skin, I do not know if there are clearly established criteria for performing and interpreting histocompatibility crossmatches. Laboratories are encouraged to contact centers where these procedures have been performed for information on the significance of crossmatch results.

7. What cross match procedures were employed?

Histocompatibility laboratories generally employ two techniques for crossmatching; complement dependent microcytotoxicity assays (CDC), with modifications to increase sensitivity, and flow cytometric assays. Each of these techniques has advantages and disadvantages which contribute to the complexity of interpreting the results, a situation which promises to become even more complicated in the future as other methodologies are added to this repertoire, e.g. ELISAs using extracted antigen.

CDC crossmatches were first introduced into the laboratory as a means of assessing the risk of hyperacute rejection in renal transplants.[51,74] Hyperacute rejection is mediated by antibody dependent complement activation which causes rapid and irreversible graft destruction.[51,103,104,107] The chief advantage of the CDC crossmatch was, and is, that it specifically detects antibodies that are capable of activating complement, that is to say, antibodies that are capable of mediating hyperacute rejection. The effectiveness of the CDC assay was evident from the earliest reports which demonstrated that approximately 80% of kidney grafts transplanted across a positive CDC crossmatch resulted in hyperacute rejection of the graft.[74] One disadvantage of the CDC assay was demonstrated by those same results, that is that about 20% of the cases ruled out by a positive CDC crossmatch would not have resulted in hyperacute rejection and that those transplant candidates may have needlessly been denied grafts. Additionally, it was apparent that the original CDC assay was either not sensitive enough or not specific enough to detect all of the relevant antibodies because a small percentage of grafts continued to be lost to hyperacute rejection.[56]

Most of the procedural modifications of CDC crossmatches that have been introduced over the years such as; separation of lymphocyte subpopulations and B cell crossmatching, adding washes prior to complement addition, increasing the length of incubations with serum and complement, and the use of anti-human globulin (AHG), have been introduced in attempts to rectify those original procedural deficiencies in specificity and sensitivity. Cytotoxicity assays have several other disadvantages in addition to sensitivity and specificity namely; the requirement for isolation of viable lymphocyte subpopulations, the subjective and time consuming evaluation process, susceptibility to technical failure due to spontaneous cell death or complement inactivation, and numerous modifications that have made standardization and interpretation complex and difficult.[12,48,110]

In an effort to address some of the problems inherent to CDC crossmatches an entirely different crossmatch procedure was developed, the flow cytometric crossmatch (flow).[14,37,76] Flow crossmatches have several distinct advantages over CDC assays. First, flow crossmatching is a more objective and quantitative method of detecting the presence of circulating alloantibody.[14,37,83] Second, while flow crossmatching still requires live cells, there is no need to physically separate lymphocyte subpopulations since that can be done electronically by the flow cytometer.[14] Third, the isotype of the

alloantibody detected can be defined by the specificity of the anti-human immunoglobulin used in the assay. And finally, flow crossmatches are more sensitive at detecting alloantibody than most, if not all, CDC crossmatches.[14,37,61,85,95,99] It was hoped that the increased sensitivity of flow crossmatches would permit identification of the small percentage of candidates that continued to experience hyperacute rejection despite the presence of negative CDC crossmatches. Unfortunately, the sensitivity of flow crossmatching, in conjunction with the fact that detection of antibody by flow cytometry bears no relation to the ability of that antibody to activate complement, creates one of the major disadvantages of flow crossmatches which is that not all positive flow crossmatches indicate a high risk of hyperacute rejection. What flow crossmatch results have been shown to correlate with, in renal and cardiac recipients, is an increased risk of acute rejection episodes.[7,14,26,49,63,73,76,85,95,101 see however 76]

One of the advantages that flow crossmatching brings to crossmatch interpretation is that the results are more objective and quantitative.[83] Roughly speaking, the further the cells are displaced to the right of the negative control the more antibody bound per cell. The more antibody bound per cell, the more likely that there will be sufficient antibody on the cell surface to activate complement, and when complement is activated hyperacute rejection or antibody-mediated graft damage can result. Antibody density on the cell surface impacts complement activation because complement factor 1 must bind two adjacent immunoglobulin (Ig) molecules simultaneously to be activated. If the density of antibody on the cell surface is so low that two antibody molecules are rarely in close proximity, the probability of complement activation is reduced. Antibody density on the cell surface depends on two factors, antigen density on the cell surface and antibody concentration in the serum. Cells with higher surface antigen expression have the potential to bind more antibody per cell and are more prone to complement induced injury. This point is supported by findings that B lymphocytes have higher class I expression per cell than T cells, and that B cells can be lysed by lower concentrations of anti-class I antibody.[14,46,75] It is important to remember though that lymphocyte MHC density is not necessarily representative of MHC density on endothelial cells or solid organ grafts and it follows that lymphocyte lysis does not always equate to graft damage.[96] However, if there is enough anti- MHC class I antibody present to mediate T lymphocyte death there is a high probability of antibody mediated graft injury as well.

The relationship between antibody titer and positive crossmatches became apparent from studies done to investigate why some candidates with a 0% CDC PRA have positive flow antibody screens or crossmatches. It has been suggested that these results could be explained if some patients make antibodies primarily of the subtypes IgG1 and IgG3, which can activate complement in humans, while other patients make primarily IgG2 and IgG4 which cannot activate complement efficiently. If this were the case, patients who make primarily IgG2 or IgG4 would have negative CDC assays and positive flow assays since the anti-human IgG antisera used in flow cytometry can detect all IgG subtypes with equal efficiency. If some patients do produce mainly IgG2 and IgG4 they would also be at low risk for antibody mediated graft damage because those alloantibodies would not effectively activate complement *in vivo* either.[77]

Using a flow T cell screening protocol and a commercial T cell CDC panel to screen a list of kidney transplant candidates, two populations of patients were defined. Both groups had positive flow screens but they differed in CDC reactivity, one group was CDC negative (flow pos/CDC neg) and the other was CDC positive (flow pos/CDC pos). The flow reactivity of the two groups was further evaluated using antibodies specific for the four IgG subtypes; IgG1,2,3 and 4. The results indicated that both groups had very similar IgG subtype profiles. In both groups the majority of T cell-binding antibody was IgG1 and IgG3, both of which are capable of complement activation. What became apparent was that the two groups differed in the amount of antibody bound per cell. In general, the median channel shift in the flow pos/CDC pos group was higher than the channel shift in the flow pos/CDC neg group. This indicated that there was more alloantibody bound per cell in the pos/pos patients than in the pos/neg patients which suggested that the difference between the two groups was not the IgG subtype of the alloantibody, but rather the difference in alloantibody titer in the patient's serum. The more sensitive flow methodology was apparently detecting lower concentrations of alloantibody, concentrations too low to activate complement even in an AHG augmented CDC assay.

This interpretation suggests that the different crossmatch protocols act as a continuum of sensitivity for detecting anti-MHC class I antibody (Fig. 1A and B). The least sensitive detection system is the T cell CDC crossmatch, followed by the B cell CDC assay,[7] the flow T cell assay,[99] with the highest sensitivity occurring in the flow B cell protocols.[57] Unfortunately, there is no clearly definable channel shift in a flow crossmatch over which all CDC crossmatches are positive and below which all CDC assays are negative. The important word here is *all*. With a cutoff set three standard deviations above the negative control *all* flow crossmatches with a channel shift below that cutoff should have negative T cell CDC crossmatches. Unfortunately, many of the crossmatches with channel shifts above that cut off would also have CDC negative crossmatches and therefore could be transplanted with little risk of hyperacute rejection. This is probably the main complaint about flow crossmatching, that too many candidates are "needlessly" ruled out by positive T cell flow crossmatches.[14,49,76]

Clearly, flow crossmatches allow an estimation of the amount of antibody bound per cell and the evidence indicates that patients with positive flow crossmatches are at increased risk for acute rejection. As discussed above, patients with a positive T cell CDC crossmatch are at high risk for hyperacute rejection.[26] If both assays are run simultaneously the results could be interpreted as follows: all candidates with a positive T cell CDC crossmatch are ruled out unless they are liver recipients, and all patients with a negative T cell flow crossmatch have very low risk of antibody mediated graft injury and can be transplanted. The patients with a negative T cell CDC assay and a positive T cell flow assay fall into a gray zone (Fig. 2) The greater the median channel shift in the flow assay the more antibody bound per cell, and the higher the risk of antibody mediated graft injury. If the antibody titer is high enough these transplants can end in hyperacute rejection. The negative side of this interpretation is that for patients who fall into that gray zone the physicians must determine how

much risk they feel is warranted in order to get these patients transplanted.[11] One advantage of running both T cell CDC and T cell flow crossmatches concurrently is that patients with positive flow and negative CDC results are identified as a population that may be transplanted with little risk of hyperacute rejection but who are at increased risk for acute rejection episodes.[49] The plus side of this situation is that immunosuppressive regimens that have demonstrated efficacy for preventing or reversing acute rejection episodes are currently available,[1-3,42,59,62,99,108,109] and new immunosuppressive agents are coming to market at a steady pace. It should be possible now to identify the population of patients who are at increased risk for acute rejection, to follow those patients closely post transplant for indicators of immune activation, and to treat those patients with regimens that will prevent or reverse the majority of rejection episodes.

There is one other concern however in relation to these patients, that being, what is the risk of these patients developing chronic rejection?[7, 81] Since one of the best indicators of patients at risk for chronic rejection is the occurrence of acute rejection, and since one factor that may contribute to chronic rejection is anti-MHC antibody,) it may be that patients transplanted across a positive flow crossmatch, who are at increased risk for acute rejection, will also be at higher risk for chronic rejection.[81, 101, 104, 106] Chronic rejection is one of the leading causes of late graft loss and none of the immunosuppressive regimens currently in use have been able to slow the rate of graft loss to this process. Each year a growing number of patients are relisted after having lost their grafts to chronic rejection. Many of these replant candidates are sensitized by the failed graft and develop broadly reactive anti-class I antibodies and quite frequently anti class II antibodies as well. These replant patients can be difficult to find crossmatch negative organs for, and they are at very high risk for losing their new grafts to hyperacute and acute rejection. With the current shortage in organs it has been suggested that patients at risk for acute rejection should not be transplanted which would allow grafts to be placed into very low risk candidates in an effort to extend the functional lifespan of all grafts.[21] This approach raises a myriad of ethical questions and dilemmas which are far too cumbersome to be addressed adequately in a chapter such as this. The jury is definitely still out on the question of how best to allocate cadaveric organs, and the debate will undoubted continue as long as there is an organ shortage and until the mechanisms of chronic rejection can be elucidated and it can be effectively treated.

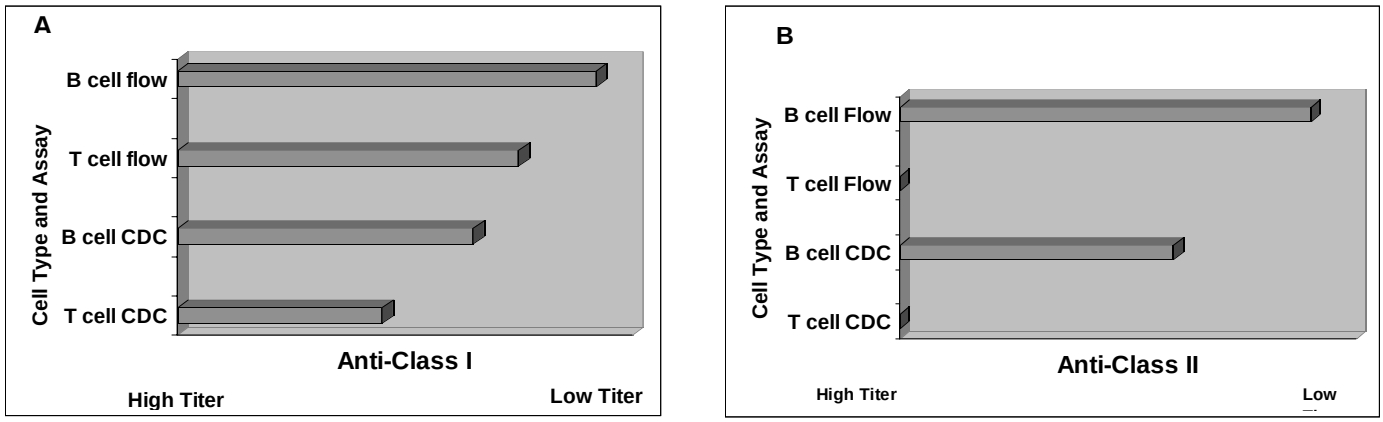


Fig. 1. Relative sensitivity of a variety of histocompatibility crossmatches. (A) The different methodologies using T or B lymphocytes as targets act as a continuum which can indicate the titer of anti-class I antibodies. (B)Anti-class II antibodies will only be detected in assays using B lymphocytes as targets unless the T cells have previously been activated and are expressing class II antigen as well.

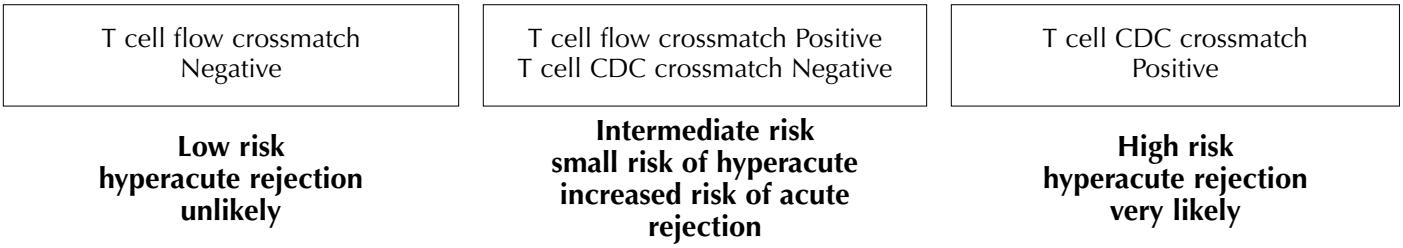


Fig. 2. Implications of T cell crossmatch results. The differences in the sensitivity of the two methods results in a gray zone where there is low risk of hyperacute rejection but some reports indicate that there is an increased risk of acute rejection and possibly increased severity of rejection episodes. The various zones reflect the anti-class I antibody titer, with a negative T cell flow result indicating very low or no anti-class I and a positive T cell CDC result indicating high titers of anti-class I antibody.

How then should the results from CDC and flow crossmatches be interpreted, and what conclusions about hyperacute rejection and graft survival can be drawn from these assays? As shown in Table 1, in the absence of autoantibody a positive T cell crossmatch is considered to indicate the possibility of anti-MHC class I antibody and a positive B cell crossmatch to indicate the possible presence of anti-class I and/or anti-class II antibody. The higher density of class I expression on B cells actually makes the B cell crossmatch a more sensitive detection system for anti-class I antibodies and B cell crossmatches are the only commonly used crossmatch that can reliably detect anti-class II reactivity.

The presence of anti-MHC class I-specific antibodies in concentrations sufficient to produce a positive T cell CDC crossmatch indicates a very high risk of hyperacute rejection in all solid organ transplants except for liver transplants. Remember though, that a positive CDC crossmatch does not "guarantee" hyperacute rejection even in renal transplants since approximately 20% of renal grafts transplanted across a positive T cell CDC crossmatch could be expected to survive.[74] This implies that in some instances allografts will succeed even against what appears to be overwhelming odds. But in an era where graft survivals of 90-95% are the norm few surgeons would be willing to take that risk and a positive T cell CDC crossmatch is universially considered the strongest single contraindication to transplantation of most solid organs.

Table 1. Various patterns of crossmatch reactivity showing relative risk of rejection and some possible interpretations.

	Cytotoxicity		Flow		Interpretation
RISK [a]	T CELL	B CELL	T CELL	B CELL	
++++	Positive	Positive	Positive	Positive	Anti class I IgG may also contain anti class II IgG. High risk of hyperactue rejection. **Do not transplant.**
?	Positive	Negative	Positive	Negative	Probably not anti class I because B cells should also be positive. Possible T cell specific antigen? Further characterization needed. ELISA or flow screens can be helpful.
++ to +++	Negative	Positive	Positive	Positive	Low titer anti class I, but can have anti class II also. Can cause hyperacute rejection if anti class II antibody is present in high titers, increased risk of accelerated acute and acute rejection, particularly in replant or sensitized candidates.
+ to ++	Negative	Negative	Positive	Positive	Low titer anti class I see above.
0 to +	Negative	Positive	Negative	Positive	Anti class II antibody, and/or very low titer anti class I, and/or IgG autoantibody. High titer anti class II may cause hyperacute rejection. May indicate increased risk of acute rejection in replant candidates and sensitized patients. Auto antibody low risk, may even be protective.
0 to +	Negative or Positive	Positive	Negative	Negative	IgM antibody, likely to be an auto antibody which is low risk. Possible low titer IgM anti class I detected early following a sensitizating event and prior to class switch to IgG. Autoabsorb to prove autoantibody and to rule out anti MHC IgM antibody.
0	Negative	Negative	Negative	Negative	No detectable anti MHC antibodies. Low risk transplant.

[a]. 0=very low risk
+=slight risk, possible increased incidence of acute rejection episodes
++=some risk of accelerated acute rejection, increased risk of acute rejection episodes
+++=moderate risk, possible hyperacute rejection, risk of accelerated acute rejection and acute rejection
++++= high risk, probable hyperacute rejection

If the T cell crossmatch is positive due to anti-class I antibody the B cell crossmatch is expected be positive as well. The significance of a positive B cell CDC crossmatch with a negative T cell CDC crossmatch is much more difficult to determine and continues to be a subject Table 1. Various patterns of crossmatch reactivity showing relative risk of rejection and some possible interpretations.for debate.[15,27] Some studies have reported that a positive B cell CDC crossmatch correlates with increased acute rejection episodes and decreased graft survival in both primary and replant candidates.[92,100] In other studies a positive B cell CDC result correlated with inferior outcome only in replant candidates,[75] and in several cases no significant effect of a positive B cell CDC crossmatch could be found.[27,46,47,73,96] This debate probably continues because of the difficulty of determining the specificity of the antibodies that can cause a positive B cell CDC crossmatch, and when all positive B cell CDC crossmatches are lumped into one group, the outcomes are so heteroge-

neous that no useful interpretation is possible. If the crossmatches are subdivided according to the specificity of the reactive antibody the interpretations are only slightly more comprehensible. If it is clear that all of the reactivity is due to autoantibody, whether IgG or IgM, it can be disregarded in all candidates. If the reactivity is due to titers of anti-class I antibody too low to be detected by a sensitive T cell CDC assay, it rarely portends a risk of hyperacute rejection but probably indicates an increased risk of complications in replant or highly sensitized patients. If the reactivity is due to anti class II antibody it represents a risk for hyperacute rejection only if it is a high titer antibody. If it is a low titer antibody, whether it is anti class I or class II it is only a contraindication to transplantation in replant candidates, highly sensitized renal patients[86,98] and in possibly in heart patients who have been sensitized. If the reactivity is due to a combination of anti-class I, class-II, and auto-antibodies the possibilities become too complex to interpret and the clinical significance of the results are essentially impossible to determine.

Whereas the significance of a positive B cell CDC crossmatch continues to be debated, there is much more agreement in regards to T cell flow results. The overwhelming consensus is that strongly positive T cell flow crossmatches indicate a risk of hyperacute graft loss in renal, heart and lung transplants. Weaker T cell reactivity in a flow assay indicates increased risk for acute rejection episodes especially in replant or sensitized patients.[7,24,26,37,49,57,63,64,73,86,95,99,101] This consensus is somewhat surprising considering that B cell CDC and T cell flow assays are similar in sensitivity (Fig. 1A) One can only surmise that the slight increase in sensitivity and the marked increase in specificity i.e., the ability to eliminate IgM interference, make the T cell flow results more clinically relevant. This suggests that in labs that have flow capability, a B cell CDC crossmatch has little, or quite possibly no, value in making clinic decisions and that it is more informative to run a T cell flow crossmatch for detection of low titer anti-class I antibodies and for eliminating interference from IgM autoantibodies.

Finally, what are the clinical implications of a positive B cell flow crossmatch when both the CDC and flow T cell assays are negative? Like the T cell flow crossmatch a B cell flow crossmatch is more sensitive, more specific, and more quantitative than the B cell CDC assay.[85] This means that the B cell flow assay is a very sensitive technique for detecting anti-class II and very weak anti-class I antibodies(Fig. 1A and B), it effectively clarifies the isotype of the antibodies, and at the same time yields a rough estimation of the titer of the anti B cell reactivity, all of which are clinically useful pieces of information. If the B cell flow crossmatch is strongly positive it infers the presence of anti-class II antibody because a large shift to the right indicates high titers of antibody and the presence of high titer anti-class I antibodies should have resulted in a positive T cell flow crossmatch as well. The presence of high titer anti-class II antibody implies some risk of hyperacute rejection but more commonly indicates an increased risk for acute rejection episodes and possibly early graft loss.[7,37,49,57,61,63,86,94] If the B cell flow crossmatch is weakly positive, whether it is anti-class I or anti-class II, it is only of concern in high risk patients such as replant candidates or patients who have a history of sensitization. In primary transplant candidates, that do not have a high PRA at the time of transplant, neither of these low titer antibodies should preclude transplantation. Even in high risk patients it is debatable if a weakly positive B cell flow crossmatch alone is sufficient reason to deny the patient a graft.[7,49,86,94 see also 37,61,85] however when the organ being grafted and other clinical information is included in the risk calculation it may be deemed too dangerous to risk transplantation with this donor. In general, a weakly positive B cell flow crossmatch appears to identify a group of patients who are at some increased risk for acute rejection episodes, a group who should be monitored closely post transplant for antibody elaboration and acute rejection. Some reports indicate that this group of patients may benefit from more vigorous immunosuppressive therapy early post transplant.[62,86,108] It appears that refusing to transplant all candidates who have antibody detectable only in flow B cell crossmatches would in some instances be an overly cautious approach that would deny organs to a number of patients who would have uneventful and successful transplants. Again, each transplant center must determine what crossmatches to perform and what the results mean for their patients. Conclusions should be based on objective analysis of the data available at their center, and the physicians who know the clinical condition of the recipient must decide if the increased risk implied by positive flow crossmatches is cause to rule out transplanting any particular candidate.[11]

Interpretation of post transplant crossmatches.

The presence of anti-donor MHC antibody post transplant has now been shown in a number of studies to correlate with an increased risk of rejection episodes and graft failure in kidney and heart transplants.[7,18,23,40,49,59,69,78,87,101] Antibodies of all three isotypes IgG, IgM and IgA, have been seen post transplant41 but at least two groups of investigators found that IgM antibodies were not detrimental to graft survival whereas IgG antibodies were.[38,87] Apparently, only donor specific antibody is associated with an increased incidence of rejection episodes,[38,58] and it appears that recipients who have anti-MHC antibodies pretransplant (PRA>10%) are at increased risk for elaborating antibody post transplant as well.[87]

Interestingly, even though circulating antibody was detectable in all of the patients in these studies, not all of the biopsies from these patients demonstrated Ig binding or complement deposition in the grafts.[54,58,87] These findings bring into question the role of antibody in the organ dysfunction and rejection in these patients. In the absence of direct evidence that the antibody was actually binding to, and directly mediating, graft injury why would the detection of antibody correlate with acute rejection? One possible explanation is that circulating antibody may be acting as a readily detectable indicator of more widespread immunologic activation, i.e., T cell activation. Acute rejection can take several different forms including accelerated acute rejection and vascular rejection, both of which may have antibody as well as cell mediated components,[2,23,54,62,108] and cellular rejection which is mediated by T cells with minimal if any Ig or complement involvement.[104] The cell mediated events occur largely in the graft, lymph nodes and spleen, and are difficult to monitor because a reliable circulating indicator of T cell activation has never been identified. B cell activation, which also occurs

in nodes and spleen, produces a soluble product which is readily detected in the circulation. This means that B cell activation is much easier to detect than T cell activation, but detecting B cell activation indirectly indicates concomitant T cell activation because T cell help is required for antibody production and class switching. When evaluating these studies, it is important not to equate statistical correlation with causation and to objectively analyze the evidence defining the actual role of humoral and cellular immunity in acute rejection episodes in recipients with circulating alloantibody post transplant.[43]

Detection of alloantibody production post transplant is subject to interference from immunosuppressive therapy and the choice of assays and interpretation of results should take this interference into consideration. The most common interference is due to residual anti-T lymphocyte preparations (ALG) which may be present in the recipient's serum post transplant, and which are lymphocytotoxic in T cell-based CDC assays.[31] For this reason flow and ELISA assays, which use species-specific secondary antibodies that do not cross-react with mouse, rabbit, or horse Ig, are often the preferred methods for post transplant testing.[93] The newer anti- IL2 receptor(IL2R) specific antibodies should be less troublesome in this regard since only a small percentage of the T cells used in panels or crossmatches are IL2 receptor positive, however, more experience with those preparations will be necessary before firm conclusions can be drawn.

Another difficulty with post transplant crossmatches is the reliance on frozen donor cells as targets. The antibody binding characteristics of cells that have been through the freeze/thaw process can be different from that seen with fresh cells. This can make it difficult to determine if increased antibody binding is a reflection of increased circulating antibody titer or simply of increased non-specific antibody binding caused by using previously frozen cells. Interpretation will be clearer if pre-transplant serum and post-transplant serum are tested simultaneously on any thawed cell preparation. Simultaneous testing on the same cell preparation makes it easier to differentiate de novo antibody production from increased non specific antibody uptake induced by cell handling.

Conclusions

This chapter has reviewed the use of donor specific-crossmatch results as a means of estimating the risk of hyperacute and acute rejection in solid organ transplantation. As stated several times throughout this chapter, interpreting crossmatch results can be very complicated and generally entails integrating information from a number of assays that use several different technologies. Experience with the protocols used at any particular institution may be important for interpreting the results, especially when dealing with cytotoxic assays where there is little standardization in methods and reagents, and where the reading of results is fairly subjective. It is reassuring however to note that consensus is routinely reached on crossmatch survey samples which indicates that the results from the majority of transplant centers must at least be comparable.

The availability of flow cytometry for crossmatching and of ELISA and flow procedures for antibody detection has lead to increased agreement on the relevance of pre and post transplant alloantibody. This may be because these techniques are more amenable to standardization, can specifically detect IgG antibody which eliminates interference from most autoantibodies, and exhibit increased sensitivity which permits detection of what had previously been subliminal antibody titers. Hopefully, these newer assays will also help resolve the debate over the relevance of a positive B cell CDC crossmatch and of anti-class II antibody. Although several studies have shown a good correlation between pretransplant PRA>10% and an increased incidence or severity of acute rejection episodes, definitive evidence of an active role for alloantibody in many acute rejection episodes is still lacking. Additionally, despite the excellent evidence in the paper by Russell et. al. for alloantibody involvement in the development of chronic rejection the effect of pre- and post-transplant PRA on chronic rejection is not clear.

Continued research in all of these areas is vital to the advancement of the field and for resolution of questions concerning the mechanism of organ dysfunction and loss. It is important that these questions be addressed in the clinic through prospective studies with adequate and appropriate control groups. One unfortunate characteristic of much of the clinical literature that was reviewed for this chapter is that a significant proportion of it is reported as case studies and retrospective studies from single institutions. While this approach can be informative, controlled studies which clearly define the criteria for acute cellular, vascular, and chronic rejection, and which have a clear definition of what constitutes a positive crossmatch should produce broadly applicable information on the implications of positive crossmatch results.

In the final analysis, crossmatch results are only one part of the total equation for estimating the risk of transplantation. A complete risk evaluation will also include consideration of the clinical risk, based on evaluation of the recipient, and donor factors. Ultimately, the final decision to proceed to transplant with any particular donor/recipient pair must be made by clinicians who have all of this information their disposal.

It is an exciting time to be involved in transplantation science as advances are made in tolerance induction, xenotransplantation, and in the understanding and treatment of chronic rejection. Such achievements will undoubtedly expand the borders of transplantation and present new challenges in our efforts to understand alloimmunity.

References

1. Ahsan N, Holman MJ, Katz DA, Abendroth CS,and Yang HC, Successful reversal of acute vascular rejection in a renal allograft with combined mycophenolate mofetil and tacrolimus as primary immunotherapy. Clin Transplantation 11: 94-97, 1997.
2. Aichberger C, Nussbaumer W, Rosmanith P, Riedmann B, Spechtenhauser B, Feichtinger H, Fend F, Pernthaler H, Ofner D, Schonitzer D and Margreiter R, Plasmapheresis for the treatment of acute vascular rejection in renal transplantation. Transplant Proc 29:169-170,1997.

3. Aichberger C, Nussbaumer W, Rosmanith P, Riedmann B, Spechtenhauser B, Feichtinger H, Fend F, Pernthaler H, Ofner D, Schonitzer D and Margreiter R, Plasmapheresis for the treatment of acute vascular rejection in renal transplantation. Transplant Proc 29:169-170, 1997. Abstract.
4. Alarif L, Snyder Tand Light JA, Transplantation of highly sensitized patients based on crossmatches using DTT-treated sera. Transplant Proc 21(1):742-744, 1989.
5. American Society for Histocompatibility and Immunogenetics, Standards for Histocompatibility Testing. Kansas City: American Society for Histocompatibility and Immunogenetics, Section I, I3.100-I3.220, 1998
6. American Society for Histocompatibility and Immunogenetics, Standards for Histocompatibility Testing, Kansas City: American Society for Histocompatibility and Immunogenetics, Section J, J1.000-J3.400, 1998.
7. Aziz S, Hassantash A, Nelson K, Levy W, Krusse A, Reichenbach D, Himes V, Fishbein D and Allen MD, The clinical significance of flow cytometry crossmatching in heart transplantation. J Heart Lung Trans 17(7):686-692, 1998
8. Baldwin WM, Soulillou JP, Claas FHJ, Peyrat MA, van Es LA and van Rood JJ, Antibodies to endothelial antigens in eluates of 88 human kidneys: correlation with graft survival and presence of T- and B-cell antibodies. Transplant Proc 13(3):1547-1550, 1981.
9. Barger BO, Shroyer TW, Hudson SL, Deierhoi MH, Barber WH, Curtis JJ, Julian BA, Luke RG and Diethelm AG, Successful renal allografts in recipients with a positive standard, DTE negative cross-match. Transplant Proc 21(1):746, 1989. Abstract.
10. Barocci S, Valente U, Carozzi S, Leprini A, Mantero D, Fontana I, Cantarella S, Millo R, Gusmano R and Nocera A, Characterization of different patterns of antibody reactivity in highly sensitized dialysis patients. Transplant Proc 20(5):951-953, 1988
11. Braun WE, Allocation of cadaver kidneys: new pressures, new solutions. Amer J Kid Dis 24(3):526-530, 1994.
12. Braun WE, Laboratory and Clinical management of the highly sensitized organ transplant recipient. Hum Immunol 26:245-260, 1989.
13. Bray RA, Cook DJ and Gebel HM, Flow cytometric detection of HLA alloantibodies using class I coated microparticles. Submitted to the 23rd ASHI Conference, October 14-17,1997, Atlanta, GA.
14. Bray RA, Lebeck LK and Gebel HM, The flow cytometric crossmatch. Dual-color analysis of T cell and B cell reactivities. Transplantation 48(5):834-840, 1989.
15. Bunke M, Ganzel B, Klien JB and Oldfather J, The effects of a positive B cell crossmatch on early rejection in cardiac transplant recipients. Transplantation 56(6):1595-1597, 1993.
16. Cantarovich F, Castro LZ, Davalos M, Laspina A, Olmos P, Saucedo G and Correa C, Incidence of previous sensitization on the results of renal transplantation. Transplant Proc 20(5):954-958, 1988.
17. Casadei D, del Carmen Rial M, Zarazaga CN and Vila N, Historical positive cross- matches in renal transplantation with living donors: An analysis of thirteen cases. Transplant Proc 21(1):745, 1989, Abstract.
18. Catalan M, Llorens R, Legarra JJ, Segura I, Sarralda A and Rabago G, Plasmapheresis as therapy to resolve vascular rejection in heart transplantation with severe heart failure: "A report of one case." Transplant Proc. 30:176-179, 1998.
19. Cerilli J, Bias W, Gerstenberger C, Clarke J and Brasile L, Clinical significance of a blood vessel crossmatch in patients with a positive current T cell crossmatch. Transplant Proc 21(1):758-759, 1989.
20. Chapman JR, Taylor CJ, Ting A and Morris PJ, The positive cross-match: antibody class and specificity correlate with graft outcome. Transplant Proc 19(1):725-726, 1987.
21. Chapman JR, Taylor CJ, Ting A and Morris PJ, Immunoglobulin class and specificity of antibodies causing positive T cell crossmatches, relationship to renal transplant outcome. Transplantation 42(6):608-613, 1986.
22. Cho YW and Cecka JM, Organ procurement organizations and transplant center effects on cadaver renal transplant outcomes. In: *Clinical Transplants* PI Terasaki, ed., UCLA Tissue Typing Laboratory, Los Angeles, pp. 427-441, 1996
23. Christiaans MHL, Overhof-de Roos R, Nieman F, van Hooff JP and van den Berg- Loonen EM, Donor-specific antibodies after transplantation by flow cytometry. Relative change in fluorescence ratio most sensitive risk factor for graft survival. Transplantation 65(3):427-433, 1998.
24. Christiaans M, Van den Berg-Loonen E, Ten Haaft A, Nieman F and Van Hooff J, Effects of flow cytometry, complement-dependent cytotoxicity and auto cross match on cadaveric renal transplant outcome. Transplant Proc 27(1):1028-1030, 1995.
25. Cicciarelli J and Terasaki PI, Sensitization patterns in transfused kidney transplant patients and their possible role in kidney graft survival. Transplant Proc. 15(1):1208-1211, 1983.
26. Cinti P, Bachetoni A, Trovati A, Berloco P, Pretagostini R, Poli L, Renna Molajoni E and Cortesini R, Clinical relevance of donor-specific IgG determination by FACS analysis in renal transplantation. Transplant Proc. 23(1):1297-1299, 1991.
27. Creemers P, Brink J and Kahn D, Interaction between panel reactive antibodies, auto- and cold reactive antibodies and a positive B cell cross-match in renal and cardiac allograft survival. Clin Transplantation 11:134-138, 1997.
28. Dalla Vecchia LK, Book BK, Milgrom ML, Jindal RM, Leapman SB, Filo RS and Pescovitz MD, Predictive value of enzyme-linked immunosorbent assay-detected IgG anti-HLA antibodies for pediatric renal allograft rejection. Transplantation 64(12):1744-1747, 1997.
29. Daly RC and McGregor CGA, Surgical issues in lung transplantation: options, donor selection, graft preservation and airway healing. Mayo Clinic Proc 72:79-84, 1997.
30. De Marco T, Damon LE, Colombe B, Keith F, Chatterjee K and Garovoy MR, Successful immunomodulation with intravenous gamma globulin and cyclophosphamide in an alloimmunized heart transplant recipient. J Heart Lung Trans 16(3):360-365, 1997.
31. Dombrausky L and Nikaein A, Removal of OKT3 from Serum. In: *American Society for Histocompatibility and Immunogenetics Laboratory Manual*, American Society for Histocompatibility and Immunogenetics, Lenexa, I.D.3.1, 1993.
32. Dowling RD, Jones JW, Carroll MS and Gray LA, Use of intravenous immunoglobulin in sensitized LVAD recipients. Transplant Proc 30:1110-1111, 1998.
33. Frost AE, Jammal CT and Cagle PT, Hyperacute rejection following lung transplantation. Chest 110:559-562, 1996.
34. Fujita S, Rosen C, Reed A, Langham MR, Howard RJ, Lauwers GY and Scornik JC, Significance of preformed anti-donor antibodies in liver transplantation. Transplantation 63(1):84-88, 1997.

35. Gaber OA, Moore LW, Schroeder TJ, Observations on recovery of renal function following treatment for acute rejection. Amer J Kid Dis 31(6 suppl 1):S47-S59, 1988.
36. Gammie JS, Pham SM, Colson Y, Kawai A, Keenan RJ, Weyant RJ and Griffith BP, Influence of panel-reactive antibody on survival and rejection after lung transplantation. J Heart Lung Trans 16(4):408-415, 1997.
37. Garavoy MR, Colombe BW, Melzer J, Feduska N, Shields C, Cross D, Amend W, Vincenti F, Hopper S, Duca R and Salvatierra O, Flow cytometry crossmatching for donor-specific transfusion recipients and cadaveric transplantation. Transplant Proc 17(1):693-695, 1985.
38. George JF, Kirklin JK, Shroyer TW, Naftel DC, Bourget RC, McGiffin DC, White- Williams C and Noreuil T, Utility of posttransplantation panel reactive antibody measurements for the prediction of rejection frequency and survival of heart transplant recipients. J Heart Lung Trans 14(5):856-864, 1995.
39. Glotz D, Haymann J, Niaudet P, Lang P, Druet P and Bariety J, Successful kidney transplantation of immunized patients after desensitization with normal human polyclonal immunoglobulins. Transplant Proc 27(1):1038-1039, 1995.
40. Greger B, Busing M, Hebart H, Mellert J, Hopt UT and Lauchart W, The development of a positive donor-specific cross-match after kidney transplantation is detrimental to the graft. Transplant Proc 21(1):750, 1989 Abstract.
41. Groth J, Schonemann C, Kadan J and May G, Dynamics of donor-reactive IgG, IgA and IgM antibodies against T and B lymphocytes early after clinical kidney transplantation using flow cytometry. Trans Immunol. 4(3): 215-219, 1996.
42. Grover FL, Fullerton DA, Zamora MR, Mills C, Ackerman B, Badesch D, Brown JM, Campbell DN, Chetham P, Dhaliwal A, Diercks M, Kinnard T, Niejadlik K and Ochs M, The past, present and future of lung transplantation. Amer J Surg 173:523-533, 1997.
43. Halloran PF, Schlaut J, Solez K and Srinivasa NS, The significance of the anti-class I response. II. Clinical and pathologic features of renal transplants with anti-class I-like antibody. Transplantation 53(3):550-555, 1992.
44. Hanto EW, Snover DC and Noreen HJ, Hyperactue rejection of a human orthotopic liver allograft in a presensitized recipient. Clin Transplantation 1:304, 1987.
45. Iwaki Y, Terasaki PI, Park MS, Heintz R. Silberman H and Berne T, Dilutions and specificity analysis of pretransplant sera. Transplant Proc 11(1):944-949, 1979.
46. Karuppan SS, Lindholm A and Moller E, Characterization and significance of donor- reactive B cell antibodies in current sera of kidney transplant patients. Transplantation 49(3):510-515, 1990.
47. Keown PA, The highly sensitized patient: Etiology, impact and management. Transplant Proc 19(1):74-78, 1987.
48. Kerman RH, Orosz CG and Lorber MI, Clinical relevance of anti-HLA antibodies pre and post transplant. Amer J Med Sci 313(5):275-278, 1997.
49. Kimball P, Rhodes C, King A, Fisher R, Ham J and Posner M, Flow cross-matching identifies patients at risk for postoperative elaboration of cytotoxic antibodies. Transplantation 65(3):444-446, 1998.
50. Kirste G, Keller J, Fischer J and Wilms H, Procedure in highly immunized patients treated with kidney transplants. Transplant Proc 20(5):949-950, 1988.
51. Kissmeyer-Nielsen F, Olsen S, Petersen VP and Fjeldborg O, Hyperactue rejection of kidney allografts, associated with pre-existing humoral antibodies against donor cells. Lancet Sept. 24,1966, p. 662-665.
52. Kobashigawa JA, Sabad A, Drinkwater D, Cogert GA, Moriguchi JD, Kawata N, Hamilton MA, Hage A, Terasaki P and Laks H, Pretransplant panel reactive-antibody screens are they truly a marker for poor outcome after cardiac transplantation? Circulation 94(suppl.II):H294-H297,1996.
53. Koep LJ, Paton BC, Terasaki PI and Starzl TE, Hyperacute rejection of a transplanted human heart. Transplantation 32: 71-72, 1981.
54. Kooijmans-Coutinho MF, Hermans J, Schrama E, Ringers J, Daha MR, Bruijn JA and van der Woude FJ, Interstitial rejection, vascular rejection and diffuse thrombosis of renal allografts: predisposing factors, histology, immunohistochemistry and relation to outcome. Transplantation 61(9): 1338-1344, 1996.
55. Laguens RP, Vigliano CA, Argel MI, Chambo JG, Rozlosnik JA, Perrone SV and Favaloro RR, Anti-skeletal muscle glycolipid antibodies in human heart transplantation as predictors of acute rejection. Comparison with other risk factors. Transplantation 65(10):1345-1351, 1998.
56. Lavee J, Kormos L, Duquesnoy RJ, Zerbe TR, Armitage JM, Vanek M, Hardesty R and Griffith BP, Influence of panel-reactive antibody and lymphocytotoxic crossmatch on survival after heart transplantation. J Heart Lung Trans 10(6):921-930, 1991.
57. Lazda VA, Identification of patients at risk for inferior renal allograft outcome by a strongly positive B cell flow cytometry crossmatch. Transplantation 57(6):964-96, 1994.
58. Leech SA, Mather PJ, Eissen HJ, Pina IL, Margulies KB, Bove AA and Jeevanandam V, Donor specific HLA antibody after transplantation are associated with deterioration in cardiac function. Clin Transplantation 10: 639-645, 1996.
59. Lindholm A, Ohlman S, Albrechtenson D, Tufveson G, Persson H and Persson NH, The impact of acute rejection episodes on long-term graft function and outcome in 1347 primary renal transplants treated by 3 cytlosporine regimens. Transplantation 56(2): 307-315, 1993.
60. Lobo PI, Spencer C, Douglas MT, Stevenson WC and Pruett TL, The lack of long-term detrimental effects on liver allografts caused by donor-specific anti-HLA antibodies. Transplantation 55(5): 1063-1066, 1993.
61. Lobo PI, Spencer CE, Isaacs RB and McCullough C, Hyperacute renal allograft rejection from anti-HLA class 1 antibody to B Cells: antibody detection by two color FCXM was possible only after using pronase-digested donor lymphocytes. Transplant Int 10:69-73, 1997.
62. Loss GE, Grewal HP, Siegel CT, Peace D, Mead J, Bruce DS, Cronin DC, Millis JM, Newell KA and Woodle ES, Reversal of delayed hyperacute renal allograft rejection with a tacrolimus-based therapeutic regimen. Transplant Proc 30:1249-1250, 1998.
63. Mahoney RJ, Ault KA, Given SR, Adams RJ, Breggia AC, Paris PA, Palomaki GE, Hitchcox SA,White BW, Himmelfarb J and Leeber DA, The flow cytometric crossmatch and early renal transplant loss. Transplantation 49(3):527-535, 1990.
64. Mahoney RJ, Norman DJ, Colombe BW, Garovoy MR and Leeber DA, Identification of high- and low-risk second kidney grafts. Transplantation 61(9):1349-1355, 1996.

65. McIntyre, JA, Higgins N, Britton R, Faucett S, Johnson S, Beckman D, Hormuth D, Fehrenbacher J and Halbrook H, Utilization of intravenous immunoglobulin to ameliorate alloantibodies in a highly sensitized patient with a cardiac assist device awaiting heart transplantation, fluorescence-activated cell sorter analysis. Transplantation 62(5):691-693, 1996.
66. McNamara D, DiSalvo T, Mathier M, Keck S, Semigran M and Dec GW, Left ventricular dysfunction after heart transplantation: incidence and role of enhanced immunosuppression. J Heart Lung Trans 15(5): 506-515, 1996.
67. Mjornstedt L, Friman S, Backman L, Rydberg L and Olausson M, Combined liver and kidney transplantation against a positive cross match in a patient with multispecific HLA-antibodies. Transplant Proc 29:3164-3165, 1997.
68. Mohanakumar T, Rhodes C, Mendez-Picon G, Goldman M, Moncure C and Lee H, Renal allograft rejection associated with presensitization to HLA-DR antigens. Transplantation 31(1):93-95, 1981.
69. Monteiro F, Buelow R, Mineiro C, Rodrigues H and Kalil J, Identification of patients at high risk of graft loss by pre- and postransplant monitoring of anti-HLA class I IgG antibodies by enzyme-linked immunosorbent assay. Transplantation 63(4): 542-546, 1997.
70. Moore SB, Ploeger NA and DeGoey SR, HLA antibody screening, Comparison of a solid phase enzyme-linked immunoassay with antiglobulin-augmented lymphocytotoxicity. Transplantation 64(11):1617-1619, 1997.
71. Nikaein A, Backman L, Jennings L, Levy MF, Goldstein R, Gonwa T, Stone MJ and Klintmalm G, HLA compatibility and liver transplant outcome: improved patient survival by HLA and cross-matching. Transplantation 58(7):786-792, 1994.
72. Noreen HJ, Interpretation of crossmatch tests. In: *The American Society for Histocompatibility and Immunogenetics Laboratory Manua*l. The American Society for Histocompatibility and Immunogenetics, Lenexa, I.C.1.1, 1993.
73. Ogura K, Terasaki PI, Johnson C, Mendez R, Rosenthal JT, Ettenger R, Martin DC, Dainko E, Cohen L, Mackett T, Berne T, Barba L and Lieberman E, The significance of a positive flow crytometry crossmatch test in primary kidney transplantation. Transplantation 56(2):294-298, 1993.
74. Patel R and Terasaki PI, Significance of the positive crossmatch test in kidney transplantation. New Eng J Med. 280(14):735-739, 1969.
75. Pellegrino M, Belvedere M, Pellegrino AG and Ferrore S, B peripheral lymphocytes express more HLA antigens than I peripheral lymphocytes. Transplantation 25:93, 1978.
76. Pelletier RP, Orosz CG, Adams PW, Bumgardner GL, Davies EA, Elkhammas EA, Henry ML and Ferguson RM, Clinical and economic impact of flow cytometry crossmatching in primary cadaveric kidney and simultaneous pancreas-kidney transplant recipients. Transplantation 63(11):1639-1645, 1997.
77. Pidwell DJ, Adams PW and Orosz CG, Immunoglobulin isotype of alloantibodies detected in flow cytometric antibody screening techniques. Presented at the 22nd annual meeting of the American Society for Histocompatibility and Immunogenetics, San Diego, CA, October 1996.
78. Puig N, Pallardo LM, Villalba JV, Sanchez J, Crespo J, Rodriguez R and Montoro J, Donor-specific flow cytometric cross-match after kidney transplantation. Transplant Proc 27(4):2369-70, 1995.
79. Reddy KS, Clark KR, Cavanagh G, Forsythe JLR, Proud G and Taylor RMR, Successful renal transplantation with a positive T-cell cross match caused by IgM antibodies. Transplant Proc 27(1):1042-1043, 1995.
80. Russ GR, McLoughney J, Nicholls C and Starr R, Monocyte alloantigens recognized by dialysis and transplant sera. Transplant Proc 20(1):17-19, 1988.
81. Russell PS, Chase CM, Winn HJ and Colvin RB, Coronary atherosclerosis in transplanted mouse hearts; II: importance of humoral immunity. J Immunol 152:5135-1541, 1994.
82. Schonemann C, Groth J, Leveren S and May G, HLA class I and class II antibodies; monitoring before and after kidney transplantation and their clinical relevance. Transplantation 65(11):1519-1523, 1998.
83. Scornik JC, Bray RA, Pollack MS, Cook DJ, Marrari M, Duquesnoy R and Langley JW, Multicenter evaluation of the flow cytometry T-cell crossmatch. Results from the American Society of Histocompatibility and Immunogenetics-College of American Pathologists proficiency testing program. Transplantation 63(10):1440-1445, 1997.
84. Scornik JC, Brunson ME, Howard RJ, et al., Alloimmunity memory and the interpretation of crossmatch results for renal transplantation. Transplantation 54:389-94, 1992.
85. Scornik J, Brunson ME, Schaub B, Howard RJ and Pfaff WW, The crossmatch in renal transplantation. Evaluation of flow cytometry as a replacement for standard cytotoxicity. Transplantation 57:621-625, 1994.
86. Scornik JC, LeFor WM, Cicciarelli JC, Brunson ME, Bogaard T, Howard RJ, Ackermann JRW, Mendez R, Shires DL and Pfaff WW, Hyperacute and acute kidney graft rejection due to antibodies against B cells. Transplantation 54(1):61-64, 1992.
87. Scornik JC, Salomon DR, Lim PB, Howard RJ and Pfaff WW, Posttransplant antidonor antibodies and graft rejection: evaluation by two color flow cytometry. Transplantation 47(2):287-290, 1989.
88. Sedmak DD and Orosz CG, The role of vascular endothelial cells in transplantation. Arch Pathol Lab Med 115:260-265, 1991.
89. Shenton BK, Bal W,Bell AE, Bookless B, Wilson SA, Healey M, Dark JH and Corris PA, The value of flow cytometric crossmatching in lung transplantation: relevance of pretransplant antibodies to lung epithelial cells. Transplant Proc 27(1):1295-1297, 1995.
90. Shroyer TW, Dierboi MH, Mink CA, Cagle LR, Hudson SL, Rhea SP and Diethelm AG, A rapid flow cytometry assay for HLA antibody detection using a pooled cell panel covering 14 serological cross reacting groups. Transplantation 59(4):626-30, 1995.
91. Shroyer TW, Diethelm AG, Deierhoi MH, Barber WH and Barger BO, Lymphocytic IgM antibody in highly sensitized (>50% PRA) autologous negative renal allograft candidates. Transplant Proc. 21(1):739-741, 1989.
92. Sirchia G, Mercuriali F, Scalamogna M, Rosso di San Secondo R, Pizzi C, Poli F, Fortis C and Greppi N, Preexistent anti-HLA-DR antibodies and kidney graft survival. Transplant Proc 11(1):950-953, 1979.
93. Susal C, Lropelin M, Groth J, Wiesel M, May G, Carl S, Staehler G and Opelz G: Protective effect of autoantibodies against the hinge region of human IgG in kidney graft recipients. Transplantation 62(10):1534-1536, 1996.
94. Talbot D, Bell A, Shenton BK, Hussein KA, Manas D, Gibbs P and Thick M, The flow cytometric crossmatch in liver transplantation. Transplantation 59(5): 737-740, 1995.

95. Talbot D, Givan AL, Shenton BK, Stratton A, Proud G and Taylor RMR, The relevance of a more sensitive crossmatch assay to renal transplantation. Transplantation 47(3):552-555, 1989.
96. Taylor CJ, Chapman JR, Fuggle SV, Ting A and Morris PJ, A positive B cell crossmatch due to IgG anti-HLA-DQ antibody present at the time of transplantation in a successful renal allograft. Tissue Antigen 30:104-112, 1987.
97. Tellis VA, Matas AJ, Senitzer D, Louis P, Glicklich D, Soberman R and Veith FJ, Successful transplantation after conversion of a positive crossmatch to negative by dissociation of IgM antibody. Transplantation 47(1):127-129, 1989.
98. Ten Hoor GM, Coopmans M and Allebes WA, Specificity and Ig class of preformed alloantibodies causing a positive crossmatch in renal transplantation. Implications for graft survival. Transplantation 56(2):298-304, 1993.
99. Thistlethwaite JR, Buckingham M, Stuart JK, Gaber AO, Mayes JT and Stuart FP, T cell immunofluorescence flow cytometry cross-match results in cadaver donor renal transplantation. Transplant Proc 19(1) :722-724, 1987.
100. Ting A and Morris PJ, Positive Crossmatch Transplants-Safe or not? Transplant Proc 13(3):1544-1546, 1981.
101. Utzig MJ, Blumke Martin, Wolff-Vorbeck G, Lang H and Kirste G, Flow cytometry cross-match. A method for predicting graft rejection. Transplantation 63(4):551-554, 1997.
102. Vaidya S, Orchard P, Haneke R and Fish J, Primary nonfunction and preformed anti- HLA antibodies. Transplant Proc 27(1):1033-1035, 1995.
103. VanBuskirk AM, Brown DJ, Adams PW and Orosz CG, The MHC and allograft rejection. In: *The Role of MHC and non-MHC antigens in allograft immunity*. T. Mohanakumar, ed. R.G. Landes company, Austin. 1994.
104. VanBuskirk AM, Pidwell DJ, Adams PW and Orosz CG, Transplantation Immunology JAMA 278(22):1993-1999,1997.
105. Van der Woude FJ, Deckers JGM, Mallat MJK, Yard BA, Schrama E, Van Saase JLCM and Daha MR, Tissue antigens in tubulointerstitial and vascular rejection. Kid Int. 48(suppl 52): S-11-S-13, 1995.
106. Van Saase JLCM, van der Woude FJ, Thorogood J, Hollander AAMJ, van Es LA, Weening JJ, van Bockel JH and Bruijn JA, The relation between acute vascular and interstitial renal allograft rejection and subsequent chronic rejection. Transplantation 59(9):1280-1285, 1995.
107. Williams GM, Hume DM, Hudson RP, Morris PJ, Kano K and Milgrom F, "Hyperacute" renal allograft rejection in man. New Engl J Med 279(12):611-618, 1968.
108. Woodle ES, Newell KA, Haas M, Bartosh S, Josephson MA, Millis JM, Bruce DS, Piper JP, Aronson AJ and Thistlewaite JR, Reversal of accelerated renal allograft rejection with FK 506 Clin Transplantation 11:251-254, 1997.
109. Woodle ES, Spargo B, Ruebe M and Charette J, Treatment of acute glomerular rejection with FK 506. Clin Transplantation 10:266-270, 1996.
110. Zachary AA, Griffin J, Lucas DP, Hart JM and Leffell MS, Evaluation of HLA antibodies with the PRA-STAT test. An ELISA test using soluble HLA class I molecules. Transplantation 60(12):1600-1606, 1995.

Crossmatches Using Solubilized Alloantigens

Patrice Hennessy, Patrick Adams, and Charles Orosz

Purpose

Since the early 1960's, the pre-transplant detection of donor-reactive alloantibodies has been accomplished by *cross-match* analysis. This test determines if transplant candidates have circulating antibodies that can bind to cells, usually lymphocytes, derived from the graft donor. Such alloantibodies have long been associated with the development of hyperacute allograft rejection. In general, alloantibodies that bind to isolated donor T cells are considered to be directed at HLA class I molecules, whereas alloantibodies that bind exclusively to B cells are considered to be directed at HLA class II molecules. These binding specificities can sometimes be demonstrated by cumbersome absorption/elution procedures, but such verification is uncommon. Alloantibodies can be directed at cell surface molecules other than MHC class I or II, and these alloantibodies are also detectable by cross-match analysis. It is not known how common these other alloantibodies are, or whether they can promote hyperacute rejection.

One approach to detect HLA-reactive alloantibodies involves isolating the MHC class I or II molecules from graft donors and testing them separately for reactivity with sera from transplant candidates. Rapid and simple techniques to solubilize MHC molecules and to capture them with MHC-reactive monoclonal antibodies have been available for many years. With these techniques, artificial cell surfaces which display only specific MHC molecules can be produced. Crossmatches performed with isolated MHC molecules greatly enhance the reliably of detecting MHC-specific alloantibodies without the inadvertent detection of alloantibodies directed at other cell surface molecules.

ELISA (enzyme linked immunosorbent assay) technology is specifically designed to detect antibodies that bind to antigens coated onto solid surfaces. To use ELISA methodology for crossmatch analysis, MHC class I molecules derived from donor blood, spleen, or lymph node are selectively anchored onto the wells of microtiter plates with murine antibodies specific for human class I MHC molecules. These wells can then be used to screen human sera for donor MHC-reactive IgG. Donor MHC-reactive alloantibodies, if present, bind to the anchored MHC antigens. These bound human alloantibodies are detected with a secondary, enzyme-linked antibody specific for human IgG (e.g., goat anti-human IgG) of high affinity and purity. Secondary antibodies are commonly linked to enzymes such as horseradish peroxidase or alkaline phosphatase. These enzymes are coupled to the Fc region of the IgG molecule, leaving the Fab regions free to bind to their specific antigen, human IgG. A colorless enzyme substrate is used to detect the binding of the secondary antibodies, which, if present, convert the substrate into a colored enzyme by-product. Thus, the production of a colored by-product in this assay indicates the presence of donor MHC-reactive alloantibodies in the serum of a transplant candidate.

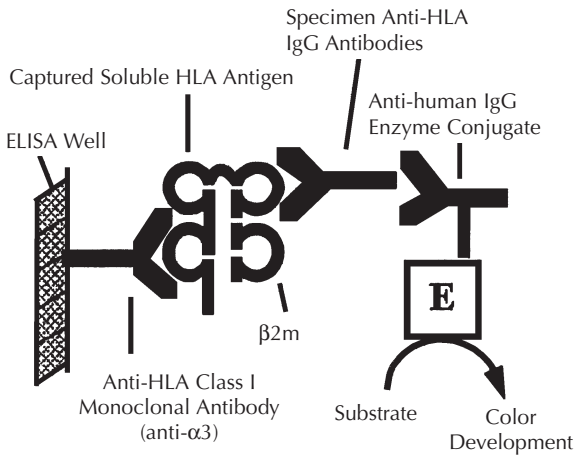


Fig.1. Principle of Donor Specific alloantibody detection by ELISA. Reprinted with permission of SangStat.

ELISA is a very sensitive method of detecting alloantibodies. It is more sensitive than routine CDC methods, and comparable to the sensitivity of flow cytometry. In addition, ELISA-detectable antibodies can be quantitated, since the intensity of colored by-product is directly proportional to the amount of alloantibody bound to the microwell surface. Finally, the ELISA assay is objective and highly reproducible, since the test results are measured photometrically with a spectrophotometer.

Specimen

1. Recipient serum specimen.
   A sterile clotted blood sample with no anticoagulant (red top tube) is required. The specimen must be properly labeled according to ASHI standards, and can remain at room temperature for 48 hours. After the tube is centrifuged at 400g for 10 minutes to condense the clot, the serum is removed, and aliquots are made and stored at 4° C for short periods of up to 7 days, or frozen at -20° C or below for extended periods. If the serum sample has been frozen, gently re-mix after thawing. Note that repeated freeze-thaw cycles of the same serum specimen should be avoided, due to possible precipitation and loss of proteins, including the alloantibodies in question.
2. Unacceptable serum specimens.
   a. Avoid specimens with reduced antibody activity such as those exposed to excess heat, vigorous agitation, repeat freeze-thaw cycles or wide ranges of pH.
   b. Avoid specimens with bacterial and fungal contamination which can deplete antibody.
   c. Avoid specimens with excessive hemolysis.
3. Donor specimen.
   Solubilized donor HLA antigens can be prepared from two different sources depending upon the availability of donor material. One source is plasma, platelets, and buffy coat spun and separated from whole blood; the other source is purified lymphocytes processed from spleen or lymph node.
4. Unacceptable donor specimens.
   a. Excessive hemolysis can release hemoglobin, which interferes with the assay.
   b. Red cell contamination can release hemoglobin into the *soluble antigen* preparation.

Reagents and Supplies

1. ELISA kits are commercially available that include all the necessary reagents to evaluate up to 100 donor-recipient pairs. These kits provide multi-well microplates pre-coated with an anti-HLA class I antibody, which permits the capture and anchoring of MHC molecules to the microwell surface. Lysis reagents used to solubilize cells and release MHC molecules are included. Also included: anti-HLA antibody positive reference, anti-HLA antibody negative reference, specimen/conjugate diluent, wash concentrate, concentrated conjugate, substrate buffer, stop solution and substrate tablets.
2. Pasteur pipettes.
3. Polypropylene tubes for specimen preparation.
4. Microfuge tubes.

Instrumentation/Special Equipment

1. Microplate reader/spectrophotometer with absorbency measurement of 490-500 nm and 600-650 nm and a 3.0 O.D. (Optical Density) minimum range .
2. Channel multichannel pipettor.
3. Centrifuge and rotor capable of holding specified tubes and reaching appropriate g forces.

Calibration

The ELISA reader and centrifuge must be calibrated according to the instrument manufacturer's instructions and must be documented. In particular, the centrifuge and rotor should be able to attain the specified speed and g force. Assays must be performed with calibrated multi-channel dispensing pipettes. Documentation of calibration is necessary. Microplate washer performance must be checked monthly and acceptable performance documented.

Quality Control

Reagents must be stored at the temperature and for no longer than the duration specified by the manufacturer. The lot numbers and optical density values of the reference reagents and controls must be recorded for each assay. These values must fall within acceptable limits for the assay to be valid. New lots of reagents must be validated by side-by-side testing with a lot known to give acceptable performance or by test with test specimen of known reactivity.

Procedure

1. Solubilization of cells:
   a. Tubes of blood are centrifuged (300 x g) to separate red cells from the buffy coat and plasma. The plasma and buffy coat are carefully removed with a pipet taking care that the red cell layer is minimally disturbed. The buffy coat layer containing primarily leukocytes is treated with lysis buffer to solubilize the cells and release the MHC molecules into solution. This solution is precipitated with an aqueous salt solution to remove unwanted proteins, and centrifuged for five minutes at 16,000 x g to pellet debris. The supernatant, which contains soluble MHC molecules (along with some additional molecules), should be clear in appearance.

   b. It is acceptable to use donor blood, lymph node or spleen and isolate lymphocytes for MHC solubilization. Lymphocytes should be isolated as per the ASHI procedures detailed in this section. Adjust the cell count according to test manufacturer's recommendation before proceeding with the cell lysis step (also see Procedure Note #1).
2. The solubilized MHC molecules are added to microwells of ELISA microtiter plates that have been pre-coated by the manufacturer with murine anti-human HLA class I monoclonal antibodies. Vigorous washing of the wells with a harsh detergent after a 60 minute incubation at room temperature, eliminates all uncaptured components of the cell lysate.
3. Recipient sera at set dilutions are added to the ELISA wells and incubated for a defined time. Excess serum components are removed by another wash step with the harsh detergent provided with the kit.
4. Horseradish peroxidase-conjugated goat anti-human IgG antibodies (or an antibody specific for another human Ig subclass) are added to the ELISA wells. These enzyme-conjugated secondary antibodies recognize and bind to any human IgG that has bound to the MHC molecules captured in the ELISA microwell. Unbound secondary antibodies are removed by another wash step with the harsh detergent.
5. Enzyme substrate solution is added to each ELISA well using a multichannel pipettor. This step is timed so that enzyme reactions are standardized.
   (Note: Do not expose the substrate solution to light).
6. After the specified reaction time, add the stop solution at the same rate of addition and sequence of wells that was used for addition of the substrate solution.
7. Using a microplate spectrophotometer, read the absorbency of each well at the designated wavelength. Optimal absorbency wavelengths differ for different enzyme reaction products. ELISA plates should be read within 10 minutes after the reaction is stopped.

Calculations

The presence and amount of specifically bound human IgG is determined by measuring the absorbency detected in wells that contain solubilized donor antigen, divided by the absorbency detected in wells lacking the donor antigens. Results are reported as a crossmatch quotient, defined as:

$$\frac{\text{Mean OD (recipient + donor)}}{\text{Mean OD (donor only) + Mean OD (recipient only)}}$$

Analysis of results can involve sophisticated computer software in which cut-off ranges for positive and negative values are determined by a crossmatch quotient.

Results

To validate the assay, wells plated with positive and negative control serum must fall within established ranges. Control wells used to determine non-specific antibody binding must also be included.

Procedure Notes

1. Acceptable alternative procedures. It is also possible to pre-incubate purified lymphocytes with test sera prior to the cell lysis step. Alloantibodies present in test sera will remain bound to the MHC molecules during cell lysis/precipitation steps, and will attach in this bound form to the captive antibodies in the ELISA microwells.
2. False negatives can result if:
   a. Components of the sera under test bind non-specifically to the coated surface of ELISA wells resulting in high background reactivity.
   b. Antibodies of differing immunoglobulin subclasses successfully compete with IgG and bind to immobilized antigen in the ELISA wells. Pre-treating serum with DTT to disrupt IgM can often eliminate this.
3. Exposure to sunlight results in substrate buffer becoming tinted in color and will increase the background readout.
4. Substrate tablets (OPD) are carcinogenic, and should never be handled without proper personal protective equipment. Prepare fresh OPD every time a test is run.
5. Thorough washing is critical. Check that each well is completely empty at the end of each wash step. If residual reagents used in previous steps remain in the wells, nonspecific binding of agents used in the subsequent step will occur, and adversely affect the results. To insure empty wells, the washed microtiter plates can be placed upside down on absorbent paper and solidly tapped several times until the absorbent paper looks dry.

Limitations of Procedure

1. Some specimens which contain only anti-MHC class I antibodies of the IgM or IgA subclasses will not be detected with the currently available commercial kits. The commercial kits cannot detect antibodies directed against non-HLA antigens.

2. Different isotypes of alloantibody can easily be detected by using a secondary conjugated antibody of the desired specificity (e.g., anti-human IgM).

References

1. ASHI Standards for Histocompatibility Testing (Adopted 4/98).
2. Kao K-J, Scornik JC, Small SJ, et. al., Enzyme-linked immunoassay for anti-HLA antibodies: An alternative to panel studies by lymphocytotoxicity. Transplantation 55:192-196, 1993.
3. Buelow R, Mercier I, Glanville L, Regan J, Ellingson L, Janda G, Claas F, Colombe B, Gelder F, Grosse-Wildr H, Orosz C, Westhoff U, Voegeler U, Monteiro F, and Pouletty P, Detection of panel reactive anti-HLA class I antibodies by ELISA or lymphocytotoxicity: Results of a blinded, controlled multicenter study. Hum Immunology 44:1, 1995.

HLA Antibody Screening and Identification by ELISA Methodology

Lori Dombrausky Osowski, Martin Gutierrez, and Beverly Muth

Purpose

ELISA metholodogy provides a cost effective, rapid and sensitive method for the detection and identification of HLA antibodies. This procedure will enumerate the steps in detecting HLA antibodies by ELISA.

Principle

For Class I HLA antibodies, pre-diluted controls and patient sera are added to the appropriate wells, allowing any antibodies to HLA Class I to bind to the immobilized HLA Class I glycoprotein. Any unbound antibody is washed away. An enzyme-labeled anti-IgG antibody is added. A second incubation allows the enzyme-labeled anti-IgG antibody to bind to any anti-HLA (IgG) antibodies that have become attached to the bound HLA antigens. Next, any unbound anti-IgG is washed away. The remaining enzyme activity is measured by adding a chromogenic substrate and reading the intensity of the color that develops. This enzyme activity is proportional to the amount of HLA-Class I antibody that is bound. The enzyme activity can also be used to calculate the panel reactive antibody (PRA) and possible antibody specificity of the patient sample in certain assays. Similar principles are applied to test for Class II antibody using appropriate immobolized target for Class II.

Note: This technology is available from several different vendors and in different sizes and tray layouts. This procedure gives principles and ideas about the general methodology, and also includes a detailed step by step procedure for a single vendor's product. Please note that the procedures are similar for Class II screening by the product described. This by no means endorses this as the only acceptable and useful product available to perform antibody identification by ELISA. If you would like information for alternative choices, please contact the first author.

Definitions

ELISA Enzyme Linked Immunosorbent Assay

QS Quickscreen™ Solid Phase ELISA (GTI, Brookfield WI) is designed to detect antibodies to HLA Class I (HLA-A, B, and C) antigens. Affinity purified HLA Class I antigens obtained from platelet pools of high numbers of Caucasian, Black, and Hispanic blood donors are immobilized in microplate wells.

QID Quik-ID™ (GTI, Brookfield WI) is a solid phase ELISA assay designed to identify specificity of anti-HLA Class I antibodies. Affinity purified HLA Class I antigens of known phenotypes are immobilized separately in micro plate wells.

Specimen

Serum obtained from one red top tube (with or without serum separating gel) is the specimen of choice. Ideally, testing should be done while the sample is still fresh to minimize the chance of obtaining false positives or false negative reactions due to improper storage or contamination of the specimen. Serum that cannot be tested immediately can be stored at 2-8°C for no longer than 48 hours or frozen (i.e. –65°C or colder). Serum should be separated from red cells when stored or shipped. Microbial contaminated, hemolyzed, lipemic or heat inactivated sera may give inconsistent test results and should be avoided.

Reagents and Supplies

1. QuikScreen Kit or Quik-ID Kit
   a. Prepared 96 well Microtiter plate with immobilized HLA Class I antigens
   b. Concentrated Wash Solution (10X)
   c. Specimen Diluent
   d. Alkaline phosphatase conjugated goat affinity purified antibody to human immunoglobulin (IgG)
   e. PNPP (p-nitrophenyl phosphate)
   f. Enzyme Substrate Buffer
   g. ELISA Stopping Solution

h. Positive Serum Control
i. Negative Serum Control
j. Plate sealers
k. Color Card

Note: Store all reagents at 2-8°C. Handle all controls in the same manner as potentially infectious material as these reagents are of human source.

2. Deionized water
3. 1.5 ml Eppendorf tubes or Blank 96 well microtiter plate for patient sample dilutions
4. 16 x 100 mm polystyrene or polypropylene tubes (or equivalent)
5. Adjustable micropipets to deliver 10-100 µl and 100-1000 µl, single or multichannel (Rainin, Finnpipet or equivalent)
6. Reagent reservoirs
7. Pipet Tips
8. Pipet aid and serological pipets
9. Graduated cylinder (one liter volume)
10. Reagent storage bottle with cap (1 liter volume)
11. Marking pen
12. absorbent towels

Instrumentation/special equipment

1. Timer
2. 37°C incubator or water bath
3. Microtiter Plate Reader capable of measuring OD at 405 nm or 410 nm with 490 nm reference and printer
4. (ELISA washer optional)

Calibration

The ELISA plate reader must be checked for OD reading accuracy on a regular basis with a control plate.

Quality Control

Daily quality control of Solid Phase ELISA is built into the test system by the inclusion of Positive and Negative Control sera. The sera must be included with each test run to help determine if technical errors or reagent failures have occurred. The new kit lot must be tested in parallel with a previously approved kit lot.

Procedure

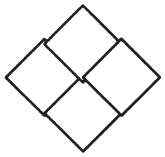
A. Preparation of Worksheets

1. Complete a GTI Reagent Worksheet (Attachment I or Ia.). Record the lot number and expiration date for the following:
   a. Master Kit
   b. Microtiter plate
   c. Wash Solution
   d. Anti-Human IgG
   e. PNPP
   f. Enzyme Substrate
   g. Stopping Solution
   h. Specimen Diluent
   i. Positive and Negative Control Sera
2. For QS, by computer or manually, complete a GTI-QS Worksheet (Attachment II). Record the following:
   a. Tech initials
   b. Date tested
   c. Plate number
   d. Lot number of kit
   e. Expiration date of kit
   f. Sample identification number for all samples tested in the appropriate alphanumeric well positions.

B. Preparation of Reagents

NOTE: Prior to use ensure that all reagents have not expired and are not turbid or contaminated.

1. Bring all reagents to room temperature.
2. Prepare Working Wash Solution by diluting Concentrated Wash Solution (10x) 1:10 with deionized water. For QID, approximately 100 ml of working wash solution will be needed for each sample to be tested. For QS, approximately 200 ml of working wash solution will be needed for each plate to be tested.
   a. Using a graduated cylinder, add 60 ml of Concentrated Wash Solution (10x) to 540 ml deionized water. Mix gently to dissolve crystals.
   b. Pour the Working Wash Solution into a one liter reagent storage bottle.
   c. Label the reagent bottle with the following information:



No NFPA Rating

Reagent: Working Wash Solution
Date Made: _____
Tech. Initial: _____
Exp. Date: 30 days from date prepared Store at: 2-8°C

C. Control Preparation

1. For QS, add 250 µl of negative control to 250 µl of Specimen Diluent Solution in an appropriately labeled tube. Gently mix the diluted control by inversion. Add 100 µl of positive control to 100 µl of Specimen Diluent Solution in an appropriately labeled tube. Gently mix the diluted control by inversion.
2. For QID, add 60 µl of Negative Serum Control to 180 µl of Specimen Diluent Solution in an appropriately labeled tube for each sample to be tested. Mix thoroughly by inversion. Add 30 µl of Positive Serum Control to 90 µl of Specimen Diluent Solution in an appropriately labeled tube for each specimen to be tested. Mix thoroughly by inversion.

NOTE: If more than one sample is to be tested, refer to Table A of Appendix I for the amounts of each control to prepare.

D. Sample Preparation

1. If necessary, thaw samples at room temperature (20°C – 25°C).
2. Prepare a serum sample dilution (1:2 QS, 1:4 QID)
   a. Gently mix thawed serum sample by inversion or aspiration and dispensing using a pipet. For QS, add 100 µl of serum sample to 100 µl of Specimen Diluent solution in an appropriately labeled 1.5 ml Eppendorf tube or a blank microtiter plate. For QID, add 600 µl of patient serum to 1800 ml of Specimen Diluent in an appropriately labeled 16x100 tube or equivalent.
   b. Gently mix each dilution.
   c. Repeat steps above for each sample to be tested.

E. Preparation of ELISA Microtiter Plate

1. Remove the microtiter plate from its protective pouch. Each QS plate will test forty samples. Label the plate with the tray/plate number for QS. Each QID plate will test two samples. Label each half of the plate with patient name, patient ID and draw date.
2. If testing an odd number of patient samples for QID, perform the following:
   a. Remove one strip of each color from the microtiter plate and reseal the remaining strips in the pouch.
   b. Save the frame of the microtiter plate after testing is completed.

NOTE: If this is the second sample to be tested from the pouch, place the strips in the frame with the colored end of the strip on top and in the order specified by the color card included in the kit.

3. Add 250-300 µl of Working Wash Solution to each well and allow to stand at room temperature (20°C – 25°C) for 5-10 minutes.
4. Decant or aspirate the contents of each well into a biohazard container. Invert plate and blot on absorbent material to remove any residual fluid.

F. Addition of Patient Samples and Control samples, QS

1. Add 50 µl of diluted positive control sample to wells G11 and H11.
2. Add 50 µl of diluted negative control sample to wells A11 through F11.
3. Test samples in duplicate by adding 50 µl of diluted sample to wells A1, A2 (B1, B2) and so on.

NOTE: Wells A12 through H12 do not contain any antigens and are to be used as "Blank Controls".

G. Addition of Patient and Control Samples, QID

NOTE: Refer to microtiter plate layout in Table B of Appendix I as a guide for the addition of patient and control sera.

1. Add 50 µl of diluted Positive Serum Control sample to well E of the orange strip.
2. Add 50 µl of diluted Negative Serum Control sample to wells A through D of the orange strip.
3. Add 50 µl of diluted patient sample to every well in all strips **EXCEPT** the orange strip. Add patient sample only to well F of the orange strip.

NOTE: Wells F, G and H of the orange strip do not contain any antigen. Wells G and H are to be used as "Blank Controls."

H. Cover plate with plate sealer and incubate plate in a dry incubator at 37°C ± 1°C for 40-45 minutes (incubator) or 30-35 minutes (waterbath).

I. Wash Step

1. Decant or aspirate the contents of each well into a biohazard container lined with absorbent material.
2. Add 200-300 µl of Working Wash Solution to all wells.
3. Decant or aspirate the contents of each well into a biohazard container.
4. Repeat steps 2 and 3 above, three more times for a total of four washes.
5. Invert plate and blot on absorbent material to remove any residual fluid.

NOTE: It is **VERY** important to completely empty each well during each washing step.

J. Preparation and Addition of Conjugated Anti-Human IgG

1. Dilute anti-Human IgG. Add 50 ml anti-Human IgG to 5.0 ml of Specimen Diluent for one QS tray (or 30 ml to 3.0 µl Specimen Diluent for one QID sample) for a 1:100 dilution. Mix well. Refer to Appendix I for reagent volumes required if testing more than one tray or sample.
2. Pour the diluted anti-IgG into an appropriately labeled reagent reservoir.
3. Add 50 µl of the diluted anti-IgG to all wells of the ELISA tray EXCEPT "Blank Controls".

K. Cover plate with plate sealer and incubate plate in a dry incubator at 37°C ± 1°C for 40-45 minutes (incubator) or 30-35 minutes (waterbath).

L. Wash Step: Repeat step I above.

M. Preparation and Addition of Chromogen

1. Prepare PNPP (P-nitrophenyl phosphate) Substrate Stock Solution by dissolving crystalline powder with 0.5 ml of deionized water. Mix thoroughly. Protect from direct light.
2. Before the end of the anti-Human IgG incubation, prepare diluted PNPP solution as follows:
   a. For each QID sample to be tested add 50 µl of PNPP Substrate Stock Solution (prepared above) to 5.0 ml of Enzyme Substrate Buffer. For each QS tray, add 100 µl to 10.0 ml. Mix thoroughly. Discard any unused portions of PNPP Stock Solution. Refer to Appendix I for reagent volumes required if testing more than one sample.
   b. Pour the diluted PNPP mixture into an appropriately labeled reagent reservoir and protect from direct light.

NOTE: Do not use the diluted PNPP if it is yellow. Prepare another dilution of PNPP.

   c. Use this reagent immediately after preparation. Discard any unused portions of PNPP Stock Solution.

3. Add 100 µl of the diluted PNPP to all the wells of the ELISA tray EXCEPT the wells A12 through H12.

NOTE: Incubation time and temperature after the addition of PNPP is critical. **DO NOT EXCEED** the recommended incubation time.

N. Incubate the tray in the dark for exactly 30 minutes at room temperature (20°C – 25°C).

NOTE: Incubation time and temperature after the addition of PNPP is critical. **DO NOT EXCEED** the recommended incubation time.

O. Turn on the microtiter plate reader at least 10 minutes before the end of the PNPP incubation.

P. Add 100 µl of Stopping Solution to each well of the microtiter tray immediately after the 30 minute incubation.

Q. Add 100 µl of deionized water to the "Blank Controls".

R. Read the absorbance (OD) of each well at 405 or 410 nm within 15 minutes of stopping the reaction. Use a reference wavelength of 490 nm. Protect the microtiter plate from light until ready to read.

S. On the microtiter plate reader printout, record the following:
Technologist's initials
Run number
Tray number
Number of washes

Results

A. Analysis / Interpretation, QS

1. Input the OD readings for the controls and samples in the **GTI QS Assay Worksheet** (Attachment II) by computer or manually.
2. If done by computer, the computer will generate all applicable calculations. If done manually, see calculations section below.
3. Patient results are expressed as one of the following: **Positive**, **Negative**, or **???** (OD values that fall outside of the acceptable range).
4. Highlight the samples with **???** and **Positive** results and repeat the QS assay for these samples.
5. Check the data to validate the run. The following criteria must be met:
   a. The positive control OD readings must be equal to or within the acceptable range values.
   b. The mean OD value for the positive control **must** be equal to or greater than 2X the mean of the negative controls.
   c. OD readings for each negative control must be within ± 20% of the mean of the negative control values.
   d. If criteria a and b are **not** met, then the run is invalid and will need to be repeated.
   e. If criterion c is **not** met, refer to Procedure Notes:A. Corrective Action. Staple the microtiter plate reader printout to the corresponding **GTI QS Assay Worksheet** (Attachment II). Staple the tape printout from the adding machine if manual calculations were performed due to computer failure. Label this printout with the date, tech. initials, and plate number.
6. Staple the microtiter plate reader printout to the corresponding **GTI QS Assay Worksheet** (Attachment II).
   a. Staple the tape printout from the adding machine if manual calculations were performed due to computer failure. Label this printout with the date, tech. initials, and plate number.
7. Route all paperwork to Supervisor/designee for review.

B. Analysis,QID

1. Double click on the appropriate icon on the desktop to open the QID software.
2. Press any key to proceed.
3. The next screen will contain the data fields listed in the steps below. Record the appropriate information followed by tab to move to the next field.
4. Tech ID field: Enter Tech initials
5. Sample Field: Use up to 8 characters to identify the sample. The computer will search for the sample ID to determine if this sample has been run before. The following will appear:

   Not Found: [Sample ID] .R00 (raw data file)
   Not Found: 1001.P00 (print file)

   Clear fields and set LOT NUMBER to [Default Lot Number] to match [Sample ID] .R00?
   (Enter/Y = Yes, Other keys = no) …

   Press Enter/Y to proceed.

6. Lot number: Enter lot number followed by "L" for left or " R" for right to indicate if the left or right side of the tray is being read.
7. Bleed Date: Enter draw date
8. Name: Enter patient name
9. Note 1/Note 2 (optional): Record in-house accession number if available.
10. Pheno: Enter patient's phenotype. This field **should** be completed if the patient's HLA typing is available. If entered, the computer will temporarily remove these antigens from the antigen file when the program performs the tail end analysis. Enter patient's phenotype with the capital letter and number of antigen with commas in between. Each antigen needs to be three characters long, as A02.

    Example: A02, A24, B07, B44, C 2, C 7

11. Skip (optional): This field functions identically to the phenotype field.
12. Press <ENTER> twice rapidly to read the sample.
13. The message: "Double check lot number then press ENTER when ready (ESC = abort)…" will display. Press <ENTER> if the lot number and side (left or right) of the tray are correct or <ESC> to modify the lot number.
14. The raw data field will appear after the reading is completed.
15. Press <ENTER>to proceed to the tail end analysis.
16. After tail end analysis is completed press <ENTER> to print the report.
17. To read another sample, return to the main menu by pressing <Esc>. The program will go back to the first screen where the information about the next sample can be entered.
18. On the microtiter plate printout, record the following information:
    Technologist's initials
    Number of Washes

C. Interpretation ,QID

1. The computer will generate a report consisting of two parts described below.
   a. The first part of the report contains the raw data. The computer will automatically calculate the cutoff value for each individual well and subtract it from the raw O.D. The resulting value is listed as the "DIFF" or difference. The reactions are listed in descending order from most positive to most negative. Any value equal to or greater than 0.000 is considered positive and is assigned an 8 by the program. Any value less than 0.000 is considered negative and is assigned a 1.
   b. The second part of the report is the tail output; which contains the panel reactive antibody (PRA) listed as "%pos" along with the results of the tail analysis.
2. Tail Output Interpretation:
   a. POS — Number of positive reactions
   b. Nzero — Number of nonzero reactions. If there are any zero (unreadable) reactions, this number will not match the number of total cells in the QID panel.
   c. %Pos — For QID this number is always the same as 468/NZ because only 1 and 8 scores are generated.
   d. 468/NZ — Percentage of panel cells with data that gave a 4, 6, or 8 reaction with the serum.
   e. 68/468 — Percentage of positive cells that had a 6 or 8 score. Always 100% for QID.
   f. 8/468 — Percentage of positive cells that had an 8 score. Always 100% for QID.
   g. ANT — Antibody specificities that show the highest correlation values.
   h. SUM — Total number of reactions that were analyzed. This number will be the sum of the ++, +-, – +, – - columns.
   i. ++ — Sera positive/antigen present on panel cells.
   j. + - — Sera positive/antigen absent on panel cell (false positive). Note that the sum of positive and false positive reactions in one line is equal to the number of false positives in the previous line. TAIL attempts to account for these false positives by assigning another specificity to the serum after dis counting the previously assigned specificity.
   k. - + — Sera negative/antigen present on panel cell (false negative).
   l. - - — Sera negative/antigen absent on panel cell.
   m. INCL — Inclusion. A number from 0.000 to 1.00 representing the number of panel cells that were positive divided by the total number of times the antigen is present on the QID panel.
   n. CORR — Correlation coefficient of the antibody specificity assigned and the serum.
   o. COMB — Combined correlation. The combined correlation of the specificity assigned plus the specificities previously assigned.
3. The OD of the positive serum control must be equal to or greater than 6X the mean of the negative serum controls. If this criterion is not met the run is invalid and must be repeated.
4. OD readings for each negative control must be within ± 20% of the mean of the negative control values. If any of these values are out of the specified range notify the supervisor/designee.
5. Route all paperwork to the Supervisor/designee for review.

D. Calculations,QS

The computer will generate calculations. In case of computer failure, data can be interpreted manually as follows:

1. Record on the **GTI-QS Manual Worksheet** (Attachment II) the following:
   a. The negative control OD readings for wells A11 through F11.
   b. The positive control OD readings for wells G11 and H11.
   c. The OD readings for the samples in the appropriate well locations.
2. Calculate the following and record in the appropriate spaces:
   a. The **Negative Control Cutoff** is equal to 2X the mean of the negative control ODs. Use the following equation where:
      N = number of negative control OD readings
      NODx = negative control OD readings with X indicating each different negative OD reading (i.e., NOD1, NOD2, etc.).

$$2 \times \left(\frac{NOD_x + NOD_x \ldots NOD_x}{N} \right)$$

   b. The **Acceptable Range** of the **Positive** control is equal to the mean of the positive control ± 20% of the mean of the positive control OD readings. Use the following equation where:
      N = number of positive control OD readings
      POD_x = positive control OD readings with X indicating each different positive OD reading (i.e., POD_1, POD_2, etc.).

$$\left(\frac{POD_x + POD_x}{N} \right) \quad - \quad 0.2 \times \left(\frac{POD_x + POD_x}{N} \right)$$

TO

$$\left(\frac{POD_x + POD_x}{N} \right) \quad + \quad 0.2 \times \left(\frac{POD_x + POD_x}{N} \right)$$

3. Check the data to validate the run as in procedure notes.
4. Calculate and record the mean OD value (SOD) of the samples tested in duplicate.

$$\left(\frac{SOD_x + SOD_x}{N}\right)$$

5. Calculate the acceptable range for the samples tested in duplicate using an adding machine with a tape printer.

$$\left(\frac{SOD_x + SOD_x}{N}\right) \quad - \quad 0.2 \times \left(\frac{SOD_x + SOD_x}{N}\right)$$

TO

$$\left(\frac{SOD_x + SOD_x}{N}\right) \quad + \quad 0.2 \times \left(\frac{SOD_x + SOD_x}{N}\right)$$

Check the results to be sure that the OD readings fall within 20% of the mean of the two values. If they do not meet this criterion, highlight the sample and re-assay.

6. Enter **Pos., Neg., or ???** in the appropriate space for each sample duplicate. Samples that show OD values equal to or greater than the Negative Cutoff value are interpreted as positive. Any sample duplicate whose OD value fall outside the acceptable range is interpreted as ???.

E. Calculations, QID

The computer will generate calculations. In case of computer failure, data can be interpreted manually as follows.

1. Record on the **QID Recording Sheet** the following:

NOTE: The Quik-ID Recording Sheet may vary between lots. Verify the lot number of the recording sheet witht he kit lot number.

a. The OD readings of the patient sera in the appropriate well location.
b. The mean of the negative control OD readings (A, B, C, and D of orange strip).
c. The positive control OD reading (E of the orange strip).
d. The OD reading of the No Antigen Well (F of the orange strip)

2. Calculate the following and record in the appropriate spaces:
   a. The Negative Control Cutoff (N.C.C.) is equal to 2X the mean of the negative control OD readings. Use the following equation where:
      N = number of negative control readings
      NOD_x =negative control OD readings with X indicating each different negative OD reading (i.e., NOD_1, NOD_2, etc.).

$$N.C.C. = 2 \times \left(\frac{NOD_x + NOD_x \ldots NOD_x}{N}\right)$$

   b. Use the Negative Control Cutoff value calculated above to calculate the cutoff value for each individual well as follows:
      Cutoff = N.C.C. X Background Adjustment Factor (BAF)*
      *The BAF values of each well can be found on the Reactivity Sheet specific for the lot number in use.
   c. Subtract the patient OD reading from the cutoff value calculated above for each well.
   d. Test results with OD values equal to or greater than the cutoff value are regarded as positive results. Test results with OD values less than the cutoff value are considered negative.
   e. Calculate the percent PRA (Panel Reactive Antibody) as follows:

$$\%PRA = \frac{\text{\# of Positive Wells}}{\text{Total \# of Valid Patient Wells}}$$

Procedure Notes

A. If one or two of the negative control values falls outside of the acceptable range:

Drop the values
Recalculate the negative control cutoff
Recheck the data

B. Additional Troubleshooting:

1. Erroneous results can occur from bacterial contamination of test materials, inadequate incubation periods, inadequate washing of test wells, or omission of test reagents or steps.
2. The presence of immune complexes or other immunoglobulin aggregates in the patient sample may cause an increased non-specific binding and produce false positives in this assay.
3. In QID, for patients with a high PRA, typically ≥80%, antibody specificity may be difficult or impossible to define. These samples may be diluted 1:2 and re-tested. The decrease in reactivity of the diluted sample may aid in the identification of the core antibody present in the patient sera.

C. Circumstances to Notify Supervisor

1. Kits containing reagents that are turbid or contaminated.
2. Failure of controls to react as expected.

Limitations of Procedure

1. This assay detects IgG antibodies reactive with HLA Class I antigens. A positive reaction indicates the presence of an HLA (IgG) Class I antibody. An IgG/M/D antibody can be substituted if requested from the vendor.
2. Some low titer, low avidity antibodies to HLA Class I antigens may not be detected.
3. Antibodies to low frequency antigens of the HLA A,B,C system may not be detected
4. Non-HLA lymphocytotoxic antibodies will not be detected. Non-IgG antibodies to HLA Class I antigens will not be detected.

References

1. Kao Kuo-Jang, Scornik Juan C. and, Small Scott J, et al. Enzyme-Linked Immunoassay For Anti-HLA Antibodies – An Alternative To Panel Studies by Lymphocytotoxicity, Transplantation 1993; 55: 192-196.
2. Natali P. G. et al, Distribution of Human class I (HLA-A, B,C) histocompatibility antigens in normal and malignant tissue of nonlymphoid origin, Cancer Res. 1984;44:4679.
3. Zinkernagel R.M. et al, MHC restricted cytotoxic T cells. Adv. Immunol. 1979;27:51.
4. Rodey Glenn E. HLA Beyond Tears, De Novo, Inc. 1991; 113.
5. Terasaki PL, Bernoco D, Park MS, Ozturk G, Iwaki Y, Microdroplet testing for HLA-A, -B, -C, and -D antigens. Am J Clin. Pathol. 1978:69:103.
6. Scornik JC. Flow cytometry crossmatch. In Zachary A, Peris G, eds. ASHI laboratory manual. 2nd ed Lenexa, KS: American Society of Histocompatibility and Immunogenetics, 1990:325.
7. Biosafety in Microbiological and Biomedical Laboratories. Center for Disease Control Negative Institute of Health, 1984 (HHS Pub. #(CDC) 84-8395).
8. GTI QuikScreen Package Insert, version 10/97
9. Bio-Tek Model EL312E Operator's Manual
10. GTI Quik-ID package insert, version 5/98
11. Quik-ID User Manual, version 11/1/99

Appendix I

TABLE A.

REAGENT AND CONTROL DILUTION CHART

Reagent	1 Plate (2 samples)	2 Plates (4 samples)	3 Plates (6 samples)	4 Plates (8 samples)	5 Plates (10 samples)
Negative Control	150 µl	250 µl	350 µl	450 µl	550 µl
Specimen Diluent	450 µl	750 µl	1050 µl	1.35 **ml**	1.65 **ml**
Positive Control	35 µl	75 µl	85 µl	115 µl	135 µl
Specimen Diluent	105 µl	225 µl	255 µl	345 µl	405 µl
Anti-IgG	50 µl	100 µl	150 µl	200 µl	250 µl
Specimen Diluent	5 **ml**	10 **ml**	15 **ml**	20 **ml**	25 **ml**
Reconstituted PNPP	100 µl	200 µl	300 µl	400 µl	500 µl
Substrate Buffer	10 **ml**	20 **ml**	30 **ml**	40 **ml**	50 **ml**

TABLE B.

TRAY LAYOUT For QID

	Y	G	B	P	R	O
A	1	9	17	25	33	N
B	2	10	18	26	34	N
C	3	11	19	27	35	N
D	4	12	20	28	36	N
E	5	13	21	29	37	P
F	6	14	22	30	38	NA
G	7	15	23	31	39	B
H	8	16	24	32	40	B
	1	**2**	**3**	**4**	**5**	**6**

N = Negative control **B = Blank**
P = Positive Control **NA = No Antigen**

Attachment I

GTI QuikScreen Reagent Worksheet

Test Performed By (date/initials)	Lot Number	Expiration Date
Master Kit		
Microtiter Plate		
Wash Solution		
Stopping Solution		
Enzyme Substrate		
Specimen Diluent		
Negative Serum Control		
Positive Serum Control		
PNPP		
Anti-Human IgG		
Reviewed By (date/initials):		

Attachment IA

GTI Quik-ID Reagent Worksheet

Test Performed By (date/initials):	Lot Number	Expiration Date
Master Kit		
Microtiter Plate		
Wash Solution		
Stopping Solution		
Enzyme Substrate		
Specimen Diluent		
Negative Serum Control		
Positive Serum Control		
PNPP		
Anti-Human IgG		
Reviewed By (date/initials):		

Tech initials:

Attachment III

Plate No: Lot No:

Date Tested:

GTI QuikScreen Manual Worksheet

Expiration Date:

Negative Control Cutoff:

Accept Range Positive:

	1	2	3	4	5	6	7	8	9	10	11
Inhouse No:											Neg. Control
OD Value: **A**											
Average/Results:											
Inhouse No:											Neg. Control
OD Value: **B**											
Average/Results:											
Inhouse No:											Neg. Control
OD Value: **C**											
Average/Results:											
Inhouse No:											Neg. Control
OD Value: **D**											
Average/Results:											
Inhouse No:											Neg. Control
OD Value: **E**											
Average/Results:											
Inhouse No:											Neg. Control
OD Value: **F**											
Average/Results:											
Inhouse No:											Pos. Control
OD Value: **G**											
Average/Results:											
Inhouse No:											Pos. Control
OD Value: **H**											
Average/Results:											

Cell Preservation

David F. Lorentzen

In this chapter, some of the important issues regarding cryopreservation are discussed.

Cell preservation is a topic which concerns, and means something different to, all who work in the area of histocompatibility testing and immunogenetics. To one it may mean a way of assuring that a blood sample mailed to the laboratory for HLA typing contains viable lymphocytes. To another, it may mean that a laboratory in Wisconsin can participate in a cell exchange and be introduced to new antigens not found in the indigenous population. To a third laboratory, cell preservation means that it can purchase a tray preloaded with a cell panel which may, in the past, have required testing several hundred individuals to characterize all specificities represented. And to a fourth lab, it means a complete panel of reference cells whose Class I or Class II DNA sequences have been characterized in laboratories around the world.

Cell preservation, *as it pertains to whole blood storage for later lymphocyte isolation*, has undergone a number of changes over the years. In general, heparinized blood requires processing within 24 hr of phlebotomy, a near impossibility in the early days of shipping blood samples for HLA testing (but becoming more commonplace today). From this was developed the Terasaki Transport Pack, which involved a preliminary separation of the white cells in a self contained unit. With the evolution of ACD (acid citrate dextrose) formula B as the preservative of choice for blood bank storage of blood, studies determined significant improvements in sample viability with the use of this anticoagulant.[1] With the use of ACD anticoagulated blood, many labs report reasonable success in serologically typing blood samples as old as two or three days post phlebotomy. For blood to be tested for T cell subsets, however, the method of storage can significantly alter the $CD3^+$ and $CD4^+$ cell populations.[2]

Cell preservation, *as it pertains to whole blood storage for later DNA isolation*, is much more forgiving than storage for serological typing. Although all of us have read about DNA analysis on 2000 year old mummies, on a piece of human hair, or on serum, samples less-than-optimally stored place restrictions on the type, number, and reliability of tests that can be performed. In addition, studies have demonstrated that heparin anticoagulant may interfere with PCR amplification,[3] and require additional steps in DNA isolation. Currently, most procedures recommend ethylenediaminetetraacetic acid (EDTA) or ACD anticoagulated blood, and allow storage of blood at 4° C for periods up to a week prior to testing.[4]

For short periods of storage within the laboratory, the simplest method is 4° C storage of the isolated lymphocytes, which often gives excellent viability for as long as three to five days. This minimizes logistic problems involved with "late Friday afternoon samples" which can be isolated and then stored over the weekend for typing on Monday. The most important potential problems in this type of storage are maintaining the proper pH of the sample and avoiding bacterial contamination. Routine addition of penicillin-streptomycin and N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffer to the storage medium, as well as handling specimens and media "cleanly" will circumvent these problems and does not require the use of "sterile" technique.

Park and Terasaki[5] and others[6] have reported excellent results in long term storage of lymphocytes at room temperature with quite good subsequent concordance of typing results in the International Cell Exchange. The major attractions of this type of storage are the relatively long "shelf life" (up to three weeks) and a considerable savings in shipping costs with the luxury of slower delivery time and insulated containers not being required. *Room temperature storage* has a number of critical demands which must be met in order to assure viability of the lymphocytes. Even more critical than in the 4° C storage, the potential for contamination with bacteria and yeasts must be minimized – this continues to be an obstacle even in the hands of the most experienced laboratories. Sterile handling is a *must*. A number of critical factors must be addressed, according to Park and Terasaki,[7] which affect the viability of the cell suspension:

1. The storage temperature should be "room temperature," which allows for considerable leeway, but should not be refrigerated or at 37° C.
2. The purity of the lymphocyte suspension should be above 95%.
3. Recommended medium is Park-Terasaki medium as described below.
4. The loss of CO_2 from the media results in the most difficult problem of long-term storage: that of pH changes. This can be circumvented with the use of HEPES buffer (pH 7.0) instead of bicarbonate buffer in the media.
5. A cell concentration of 2×10^6 appears to be optimal for storage of the lymphocytes in 0.4 ml microcentrifuge tubes.
6. Spacing between the cells appears to affect the lymphocyte storage with best results obtained using a 0.4 ml microcentrifuge tube. Cells should be allowed to settle into the pointed bottom of the tube during storage.
7. Medium should be replenished every 7 days by removing the old medium and adding fresh.

Park-Terasaki Medium (modified McCoy's Medium) can be made as follows:

1. Mix the following:
   a. McCoy's powder without $NaHCO_3$ 6.5g
   b. Antibiotics:
      –penicillin 100,00 units
      –streptomycin 0.1 g
      –gentamicin 8 mg

c. Fetal bovine serum (FBS) 2.5 ml
d. pH indicator (0.5% Phenol Red) 3.0 ml
e. Double distilled water 450 ml

2. Adjust pH to 7.0 with 5N NaOH
3. Add 6 ml of HEPES buffer solution from the following formula:
   –HEPES powder 23.83 g
   –Distilled water 100 ml
4. Add double distilled water to a total volume of 500 ml.
5. Filter with a 0.22 µ filter.

Cryopreservation of lymphocytes provides excellent long-term storage without many of the concerns involved in maintenance of pH or bacterial contamination of room temperature storage. Two major techniques are in common use for long-term cryopreservation, including controlled-rate freezing and stepwise freezing with initial freezing at -70° C with transfer to liquid nitrogen.

Lymphocyte cryopreservation requires pre-treatment of the cells with a cryoprotectant agent, commonly dimethylsulphoxide (DMSO). Without the use of this agent, lymphocytes can undergo damage by several mechanisms. Cooling too slowly results in cell damage by shrinkage due to intracellular water loss and ensuing high salt concentration,[8] while cooling too quickly causes destruction of the cells resulting from the formation of ice crystals within the cells. The inclusion of DMSO in the freezing solution, together with relatively slow cooling rates (typically -1° C/min for lymphocytes), serves to reduce the size of the intracellular ice crystals as well as the concentration of solutes.

The benefit of the cryoprotectant is not without its own set of difficulties. DMSO transfers quite slowly into and out of the cells[9] and care must be taken to slowly add freezing or thawing medium to the cell suspension to reduce the osmotic stress on the cells. Additionally, DMSO demonstrates toxicity to lymphocytes at warmer temperatures so care must be taken to keep samples cool and minimize the time which cells are exposed to the cryoprotectant, both prior to freezing and following thawing of the sample.

Freezing lymphocytes on trays presents its own set of problems, probably the greatest of which is the inability to slowly review the DMSO from the cells, due to the minimal volume in the wells of the tray. This results in a much shorter "shelf life" of usually six months or less as compared with storage measured in years for lymphocytes cryopreserved in bulk. In addition, viability may be considerably diminished in tray frozen lymphocytes, with no good means available to remove cells killed in the freeze-thaw procedure. Nonetheless, lymphocytes frozen in trays can provide a quick and effective means of rapidly identifying antibodies in patient sera.

Cryopreservation of samples for future DNA-based typing requires less stringent handling procedures than those utilized for lymphocytotoxicity. Some laboratories freeze whole blood samples directly in the vacuum tubes in which the blood was drawn (use a -20° C freezer – don't try this in a -80° C freezer or the glass tubes will shatter), others remove the buffy coat, lyse the red blood cells and freeze the WBCs either in solution or "snap freeze" the dry pellet. The variations in cryopreservation for DNA testing are much more numerous than for cytotoxicity, due to the fact that viable lymphocytes are not a requirement.

Using appropriate methodology, lymphocytes can be stored frozen for many years with subsequently thawed cells having splendid viability. Short-term cryopreserved lymphocytes also retain cell surface markers useful in determination of T cell and large granular lymphocyte subsets using flow cytometry.[10,11]

References

1. Moore SB, Beckala H, DeGoey S, and Leavelle D, A Report on the use of ACD (Solution B) as whole blood transport medium for recovery of lymphocytes for HLA typing. In: *The AACHT Laboratory Manual*: AA Zachary and WE Braun, eds.; The american Association for Clinical Histocompatibility Testing, New York, pI-27-1, 1981.
2. Huang HS, Su IJ, Huang MJ, The effect of blood storage on lymphocyte subpopulations. Chung Hua Min Kuo Wei Sheng Wu Chi Mien I Hsueh Tsa Chih 20(1):46, 1987 (abstract in English).
3. Beutler E, Gelbart T, Kuhl W, Interference of heparin with the polymerase chain reaction. Biotechniques 9:166, 1990.
4. "Procedure for Sample Collection, Shipping, and Storage of HLA-DR/DQ DNA Samples," November 23, 1992, National Marrow Donor Program.
5. Park MS, Terasaki PI, Storage of human lymphocytes at room temperature. Transplantation 18:520, 1974.
6. Bernoco D, Perdue S, Terasaki PI, Loon J, Park MS, International Cell exchange. Transplantation Proceedings 10:717, 1978.
7. Park MS, Terasaki PI, Human lymphocyte preservation at room temperature. In: *NIAID Manual of Tissue Typing Techniques, 1976-1977*; JG Ray, DB Hare, PD Pedersen, and DI Mullally, eds.: DHEW Publication No. (NIH) 76-545, Bethesda, p201, 1976.
8. Lovlock JE, The mechanism of the protective action of glycerol against haemolysis by freezing and thawing. Biochem Biophys Acta 11:28, 1953.
9. Strong DM, Cryobiological approaches to the recovery of immunological responsiveness to murine and human mononuclear cells. Transplantation Proceedings 8:203, 1976.
10. Prince HE, Lee CD, Cryopreservation and short-term storage of human lymphocytes for cell surface marker analysis. Comparison of three methods. J Immunological Methods 23;93(1):15, 1986.
11. Jones HP, Hughes P, Kirk P, and Hoy T, T-cell subsets: effects of cryopreservation, paraformaldehyde fixation, incubation regime and choice of fluorescein-conjugated anti-mouse IgG on the percentage positive cells stained with monoclonal antibodies. J Immunological Methods 27;92(2):195, 1996.

Cryopreservation of Lymphocytes in Bulk

D. Michael Strong

Principle/Purpose

In the past, the histocompatibility laboratory has used cryopreserved lymphocytes primarily for lymphocytotoxicity assays such as typing and antibody screening or mixed lymphocyte cultures. With the advent of molecular biology techniques and flow cytometry, these reagents are being used more frequently in other procedures. Use of frozen thawed cells in functional assays or in assays for determination of cell surface markers require closer attention to technique than does the lymphocytotoxicity assay.[3] Cryopreservation and long term liquid nitrogen storage can affect the expression of cell surface determinants and also functional activities of mononuclear cells.[11,12,13,2] Several laboratories have reported that, under certain conditions, selection of lymphoid subsets may occur following freezing and thawing.[1,6] Furthermore, lymphoid clones and B- or T-lymphoblastoid cell lines (LCLs) have different optimum cooling rates.[10] Although sophisticated controlled rate freezing devices are not absolutely required for lymphocyte cryopreservation, such equipment usually increases reproducibility and improves recovery.[5] Of great importance is the quality of the cell preparation itself and the handling of cells prior to and following freezing and thawing.

Since the early discovery of the cryoprotective properties of glycerol, a great deal of investigation has gone into the determination of the mechanisms of freezing injury.[9] Briefly, cells that are cooled too slowly, to below freezing temperatures, are damaged by the resulting increase in cell concentration and cellular shrinkage which occurs as water is removed during the formation of extracellular ice.[7] Conversely, if cooling is too rapid, a new mechanism is invoked in which shrinkage no longer occurs but the cell is damaged by the formation of intracellular ice, either during freezing or upon thawing.[8] Cryoprotectants such as dimethylsulfoxide (Me_2SO), reduce the amount of ice present during freezing and reduce solute concentration thus reducing ionic stress. However, these compounds can themselves cause osmotic injury since they are hypertonic and can cause damage during their addition or removal. Optimum cooling rates vary from cell type to cell type depending on differences in membrane permeability and intracellular water which is removed during the dehydration phase of slow cooling and extracellular ice formation. In addition, not only is the redistribution of solute during freezing a potential source of damage, but ice/cell interactions are also.[4] In general, the larger the cell volume, the slower the rate of cooling to allow equilibration of intra- and extra-cellular water during freezing.

Specimen

Cells can be prepared from a variety of sources including lymph nodes, spleen or peripheral blood drawn in heparin, or acid citrate dextrose (ACD). Cell preparations with low viabilities prior to cryopreservation will result in poor recovery. It is preferable to isolate mononuclear cell preparations free of platelet and granulocyte contamination. Cell suspension should be maintained in tissue culture medium such as RPMI 1640 or McCoy's 5A containing 10% serum, either fetal calf serum, pooled human serum, or autologous serum. Cell survival tends to be better at room temperature, however cells can be stored at 4° C if it is required for them to be stored for a longer periods of time in order to avoid contamination. It should be noted that some functional assays may as well as cell determinate assays be affected by storage at 4° C.

Reagents and Supplies

1. Dimethylsulfoxide, Sigma, St. Louis, MO
2. RPMI 1640, GIBCO/Life Technologies, Rockville, MD
3. McCoy's 5A, GIBCO/Life Technologies, Rockville, MD
4. Fetal calf serum, HyClone, Logan, UT
5. DNAse stock solution, Sigma, St. Louis, MO

Cryoprotective Medium

1. Dimethylsulfoxide (Me_2SO) in serum free medium (RPMI 1640, McCoy's 5A, or other culture media) to achieve a 15% volume/volume concentration.
2. Prepare the solution fresh for each freezing procedure and cool to 4° C before adding to cells.

Thawing Medium

1. RPMI 1640, McCoy's 5A or other culture media containing 10% fetal calf serum (FCS), pooled human serum (PHS), or autologous serum, warmed to room temperature (RT).

DNAse Stock Solution

1. Deoxyribonuclease type 1: Add 4800 units deoxyribonuclease/ml of water. Dispense into microtubes and store at -70° C.

Instrumentation/Special Equipment

1. Cryomed Model 1010, Forma Scientific, Inc., Marietta, OH
2. Gordinier, Gordinier Electric, Roseville, MI
3. Mr. Frosty, Nalge Nunc International Corporation, Rochester, NY

Calibration

All controlled rate freezing devices will need to be calibrated to obtain the appropriate cooling rates. This is done empirically using the sample preparations one expects to use in the laboratory and developing cooling curves to achieve optimum recoveries. These are programmable devices and once the program is determined, they are then preset to be reproducible.

Quality Control

1. Thermocouple control: It is preferable to use a cell sample made up identically to samples being frozen. Alternatively, an equal volume of medium containing serum and Me_2SO of the same concentration can be used. Control rate freezing devices generate control charts that can be saved to provide a record of the freezing process.
2. Thermal exposure indicator: A good control for monitoring freezing temperatures and shipment is "Cryoguard-70" (Controlled Chemicals, Ann Arbor, MI). This is a colored solution that can be calibrated to be activated at the temperature of storage and will remain green as long as that temperature is maintained. The indicator becomes irreversibly pink to red within approximately two hours when the environment exceeds this preset temperature of storage.

Procedure

Controlled Rate Freezing

1. Isolate lymphocytes from whole blood using a standard density gradient method.
2. Suspend cells in medium containing 20% FCS, PHS, or autologous serum.
3. Examine cell suspension for purity and adjust concentration to twice that of the desired final concentration.
4. Make up the cryoprotective medium and cool to 4° C.
5. Label the freezing ampules with the name of the donor, the cell concentration, and the date.
6. Turn on the controlled-rate freezer and bring the chamber to 0° C.
7. Cool the cell suspension to 4° C, and slowly add an equal volume of the cryoprotective medium to the cell prep with constant mixing. Both solutions should be kept at 4° C to avoid toxicity.
8. Dispense immediately into vials for freezing.
9. Place vials into the chamber of the freezer, insert the "sample temperature" thermocouple into one vial, and bring the temperature of the samples to chamber temperature (0° C).
10. When sample and chamber temperatures have equilibrated, begin the program following the instructions of the manufacturer.
11. Cool samples at 1° C/min to -30° C and 5° C/min to -80° C. The program should compensate for the latent heat of fusion (where the sample freezes) so that the cooling curve remains linear.

    The appropriate program settings must be achieved by trial and error in order to obtain a relatively smooth curve. Programs will need to be adjusted with changes in volume, container, or Me_2SO concentration.
12. When samples have reached -80° C, quickly transfer to liquid nitrogen storage (vapor phase). Do not allow samples to be exposed to RT for more than a few seconds. If samples are frozen in glass ampules, cool to -100° C before transferring to storage.

Freezing Without Controlled Rate Equipment (step-wise)

1. The procedure remains the same except that freezer vials are placed into a Styrofoam box at the bottom of an ultra-low freezer (-70 to -80° C) or in the vapor phase at the top of a liquid nitrogen freezer for 2-24 hrs prior to transfer to nitrogen storage. Cells stored at -70 to -80° C will remain viable for several months depending on fluctuations of temperature in the freezer. The Nalge Nunc International Corporation provides a freezing unit (Mr. Frosty) that is economical and can be placed in a -80° C freezer to achieve 1° C/min cooling.

2. Since conditions may vary from freezer to freezer and within each freezer, several trial attempts may be needed to determine the exact place in which the vials must be placed to achieve the best recovery.
3. An alternative step-wise method of freezing can be achieved by placing the vials in a special Styrofoam plug designed to fit into the neck of a liquid nitrogen container. Place the plug containing the vials into the container and allow to cool for 30 min prior to transfer into storage phase. Several experiments may be necessary to determine the length of time and the depth at which to place the container to obtain optimum results.

Thawing and Washing of Frozen Cells

1. Warm tissue culture medium containing 10% FCS, PHS, or autologous serum, to RT or 37° C.
2. Remove vial of cells from freezer and place immediately in a 37° C water bath, mixing as the sample thaws.
3. As the last ice crystal is about to melt, remove the cap and add the thawing solution drop-wise to the cells, mixing continuously until the freezing vial is full.
4. Transfer the contents of the vial to a 15 ml tube and begin adding additional medium drop-wise with constant mixing.
5. When the tube is approximately half full, medium can be added more rapidly to fill the tube.
6. Centrifuge the tube at 150 x g for 10 min. Pour off supernatant and gently resuspend the cell button before adding a known volume of medium.
7. Count cells, check viability, and adjust concentration as desired.

Calculations

N/A

Results

This procedure should routinely yield greater than 80% recovery of lymphocytes as determined by cell count and viability testing. Depending on the assay being employed by the laboratory, results should also be established for each independent assay to determine optimum criteria.

Procedure Notes

If viability is poor, dead cells can be removed by Ficoll-Hypaque (FH), Percoll or bovine serum albumin (BSA) gradients or DNAse treatment.

FH Gradient

1. Using Fisher tubes, layer cell prep over 0.5 ml FH gradient.
2. Spin in Fisher centrifuge at 2500 x g for 5 min.
3. Using Pasteur pipette, remove lymphocyte layer at FH interface.
4. Transfer to clean Fisher tubes. Fill tubes with clean medium containing serum and mix. Centrifuge at 1000 x g for 1 min and remove supernatant.
5. Resuspend cells, count and test viability. Standard size (15 ml) gradient tubes may also be used to remove dead cells.

Dead Cell Removal by DNAse Treatment

1. Add 200 ml of DNAse stock solution to each ml of cell suspension containing 5 x 10^6 PBMC.
2. Mix and incubate for 5 min at 37° C.
3. Wash twice in medium containing 10% serum.

Precautions

The handling of cells prior to and following freezing and thawing is at least as important as the freezing itself. The following precautions are important in obtaining optimum recovery of cells following freezing and thawing and can be used to review procedures when problems occur.

1. Obtain mononuclear cell preparations free of platelet and polymorphonuclear cell (PMN) contamination.
2. Use small aliquots (0.2-2 ml) containing a minimum of 2 x 10^6 cells/ml.
3. Maintain cell suspension in greater than 10% serum at all times.
4. Control the cooling rate at approximately 1° C/min, not to exceed 5° C/min, to -30° C. These cooling rates can also be achieved by step-wise freezing as described above.
5. Store in the vapor phase of liquid nitrogen. Storage at -80° C will result in shorter life span of the frozen cells. Storage in liquid can result in cross-contamination.
6. When transferring cells in the frozen state, do not expose cell suspensions or antisera to the CO_2 vapors of dry ice for any extended period of time and do not allow the temperature of the sample to rise above -60° C. Repeated thawing and refreezing will damage cells.

7. Thaw the cell suspension rapidly in a 37° C water bath with constant mixing.
8. Use a slow or dropwise dilution of the cell suspension with RT or warmer medium containing serum to allow for osmotic equilibrium. The careful handling, slow centrifugation, and resuspension of cells prior to dilution and assay are important in assuring optimum cell recovery.

Common Variations

1. There are a variety of freezing containers which have been demonstrated to be adequate for the freezing and storage of lymphocyte suspension. These include Nunc vials, Beckman vials, glass vials, straws, and Terasaki trays.
2. A final concentration of 10% Me_2SO is often employed in step-wise freezing procedures.
3. Frozen lymphocytes can be stored at -80° C for periods from one to two years. Adequate recovery of cells may vary, however, depending upon the frequency with which the freezer is opened and the variation in temperature that may occur. Cells stored below -100° C (nitrogen vapor phase) can be stored indefinitely.

Limitations of Procedure

Recovery of lymphocytes following freezing and thawing is dependent on the quality of the cell preparation that is used at the beginning of the procedure. Also, this can be effected by storage conditions, particularly if freezers are frequently entered and racking systems being removed to take out samples. This results in thawing and refreezing of samples over time that may result in gradual loss of viability. Technical staff should be instructed to take care about exposing samples to room temperature for any lengths of time to assure adequate low temperature storage.

References

1. Farrant J, Knight SC, Morris GJ, Use of different cooling rates during freezing to separate populations of human peripheral blood lymphocytes. Cryobiology 9(6):516-525, 1972.
2. Fiebig EW, Johnson DK, Hirschkorn DF, Knape CC, Webster HK, Lowder J, Busch MP, Lymphocyte subset analysis on frozen whole blood. Cytometry (4):340-350, 1997.
3. Gjerset G, Nelson K, Strong DM, Methods of Cryopreservation of Cells. In: *Manual of Clinical Laboratory Immunology*, Fourth Edition; NR Rose, EC deMacario, JL Fahey, A. Freidman, GM Penn, eds; Am. Soc. Micro., Washington, D.C.; 61-67, 1992.
4. Hubel A, Cravalho EG, Nunner B, Körber C, Survival of directionally solidified B-lymphoblasts under various crystal growth conditions. Cryobiology 29:183-198, 1992.
5. Ichino Y, Ishakawa T, Effects of cryopreservation on human lymphocyte functions: Comparison of programmed freezing method by a direct control system with a mechanical freezing method. J Immunol Methods 77:283-290, 1988.
6. Knight SC, Farrant J. Morris GJ, Separation of populations of human lymphocytes by freezing and thawing. Nature (New Biol) 239:88-89, 1972.
7. Lovelock JE, The haemolysis of human blood cells by freezing and thawing. Biochem. Biophys. Acta. 10:414-426, 1953.
8. Mazur P, Farrant J, Leibo SP, Chu EHY, Survival of hamster tissue culture cells after freezing and thawing. Interactions between protective solutes and cooling and warming rates. Cryobiology 6:1-9, 1969.
9. Polge C, Smith AU, Parkes AS, Revival of spermatozoa after vitrification and dehydration at low temperatures. Nature 11:28-36, 1949.
10. Strong DM, LaSane F, Neuland CY, Cryopreservation of lymphocytes and lymphoid clones. In: Developments in Industrial Microbiology; L Underkofler, ed.; Soc Ind Micro; Arlington, VA; Vol. 26; 655-665, 1985.
11. Strong DM, Ortaldo JR, Pandolfi F, Maluish A, and Herberman RB, Cryopreservation of human mononuclear cells for quality control in clinical immunology. I. Correlations in recovery of K- and NK-cell functions, surface markers, and morphology. J. Clin. Immunol. 2:214-221, 1982.
12. Tollerud DJ, Brown LM, Clark JW, Neuland CY, Mann DL, Pankiw-Trost LK, Blattner WA, Cryopreservation and long-term liquid nitrogen storage of peripheral blood mononuclear cells for flow cytometry analysis: effects on cell subset proportions and fluorescence intensity. J. Clin. Lab. Analysis 5:255-261, 1991.
13. Venkataraman M, Effects of cryopreservation on immune responses: VII. Freezing induced enhancement of IL-6 production in human peripheral blood mononuclear cells. Cryobiology 31:468-477, 1994.

Contact Information:

D. Michael Strong, PhD, BCLD
Puget Sound Blood Center
921 Terry Avenue
Seattle, WA 98104
Telephone: (206) 292-1889
FAX: (206) 292-8030
email: dmstrong@psbc.org

Cryopreservation of Lymphoblastoid Cell Lines

Soldano Ferrone

Purpose

To maintain Lymphoblastoid cell lines (LCLs) for many years. It is advisable to cryopreserve LCLs as soon as they are transformed or adequate numbers are obtained in order to avoid contamination, especially with mycoplasma. LCLs can easily be frozen without a temperature-controlled-rate freezer.

Reagents and Supplies

Cryopreservation Solution

1. RPMI 1640 medium with antibiotics
2. Fetal calf serum (FCS) 10% [volume (v)/v]
3. Dimethylsulfoxide (DMSO) 25% (v/v)

Chill the solution on ice.

Instrumentation

1. Centrifuge
2. Liquid nitrogen freezer

Procedure

1. Pellet lymphoblastoid cells by centrifugation at 800 x g for 10 min and resuspend in one volume of FCS and one volume of ice cold, freshly prepared cryopreservative solution to a final concentration of 1 x 10^7 cells/ml.
2. Dispense 1 ml aliquots of cell suspension into 2 ml screw-cap plastic vials.
3. Incubate vials overnight in gas phase of liquid nitrogen (-70° C) or in a -70° C Revco freezer and then transfer them to the liquid phase of nitrogen.
4. Transfer samples to be thawed promptly from freezer to a waterbath set at 37° C.
5. When the last ice in the vial has melted, transfer cell suspension to 10 ml of culture medium warmed to 37° C.
6. Collect cells by centrifugation and transfer them to 10 ml of medium in a culture flask. After overnight incubation, check cells for viability and culture them as usual.

Procedure Notes/Troubleshooting

Contamination may be the major problem. To avoid contamination, reagents must be prepared under aseptic conditions and filtered using 0.2 m filter. DMSO may be used without filtration since no organism can survive in it.

References

1. Strong DM. Cryobiological approaches to the recovery of immunological responsiveness to murine and human mononuclear cells. Transplantation Proceedings 8:203,1976.
2. Prince HE, Lee CD. Cryopreservation and short-term storage of human lymphocytes for cell surface marker analysis. Comparison of three methods. J.Immunological Methods 23;93(1):15, 1986.

Cryopreservation of Lymphocytes in Trays

Donna L. Phelan

Purpose

Cryoprotective agents, such as glycerol and dimethylsulfoxide (DMSO), provide for long-term storage of different types of cells by minimizing the detrimental effects of the freezing process. Cells that are cooled too slowly are damaged by the resulting high salt concentration and cellular shrinkage which occurs as a result of water being removed as ice is formed. Alternatively, if cells are cooled too rapidly, cells are damaged by the formation of intracellular ice crystals. Cryoprotectants reduce salt concentration at any temperature, prevent intracellular ice formation and protect cell membranes against irreversible denaturation. Considerations to be taken when choosing the appropriate protective additive are cell types, cooling rates and various levels of physiological function.

Lymphocytes frozen directly in microtest trays are useful for screening small numbers of serum samples, such as the monthly screening of dialysis patients' sera on a routine daily basis. They are also necessary for the performance of STAT antibody screens on potential heart transplant recipients or platelet transfusion patients.

In this technique, cells from a total of 6 subjects are frozen on a 72 well tray providing sufficient wells for the testing of 12 serum samples. One cell prep is added to each of the 6 lettered (A-F) rows and each serum specimen is added to each of the 12 numbered (1-12) rows. In the routine monthly screening procedure, each serum is tested against a panel of 36 cells which have been well characterized for HLA antigens. Each specificity of the HLA-A, B and DR loci is represented at least twice in the panel. Therefore, a full set of screening cells involves 6 trays, each containing 6 cells for a total of 36 cells. Twelve sera can be studied for each cell set.

Specimen

Heparinized blood: 50-60 ml per cell donor.

Reagents and Supplies

1. Carbonyl Iron
2. Lymphocyte separation medium (LSM)
3. Lympho-Kwik™ (One Lambda, Inc.)
4. Human Serum
5. DMSO
6. McCoy's 5A Medium, 500 ml
   a. N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) 12.5 ml
   b. AB Serum 50.0 ml
   c. gentamicin (50 mg/ml) 0.5 ml
   pH to 7.2.
7. FITC 1:10 dilution
   a. 1 vial FITC [Goat anti-human IgM (whole molecule)]
   b. deionized water 2 ml
   c. RPMI-azide 18 ml
8. Ethidium Bromide – Stock Solution
   a. ethidium bromide 0.5 g
   b. deionized water 5 ml
9. Ethidium Bromide – Working Solution
   a. stock ethidium bromide 2 ml
   b. RPMI-azide-EDTA 5 ml
10. RPMI-azide
    a. sodium azide 0.02 g
    b. HEPES 2.5 ml
    QS to 100 ml with RPMI and pH to 7.2.
11. RPMI-azide-EDTA
    a. EDTA 5 g
    QS to 100 ml with RPMI-azide.

12. Freeze Media
    a. L-glutamine 1.0 ml
    b. gentamicin 0.1 ml
    c. HEPES 1.0 ml
    d. AB serum 10.0 ml
    QS to 100 ml with RPMI-azide.
13. DMSO-Freeze Media
    a. Freeze Media 17.2 ml
    b. DMSO 2.8 ml
    Add DMSO dropwise: approximately 1 ml/min.

Instrumentation

1. Controlled rate freezer
2. 37° C Incubator
3. Microcentrifuge

Procedure

Lymphocyte Preparation

This technique is designed for antibody screening by two color fluorescence, thus it describes the preparation of mixed lymphocytes with FITC-labeled B cells. The freezing technique can be used with any lymphocyte population, i.e., mixed unlabeled, T cells or B cells prepared by any of the various methods. Modifications for cell preparations other than FITC-labeled B cells are in parenthesis.

1. For each of six donors:

Label	Number
50 ml conical tubes	3
16 x 100 glass tubes	21
16 x 95 plastic tube	1
12 x 75 plastic tube	1
1 ml microcentrufuge tubes	12

2. Draw blood from each donor.
3. In each conical tube, place 15 (6 mm) glass beads, 2 large scoops (700 mg) carbonyl iron, 8 ml methyl cellulose, 15 ml McCoy's and 16 ml of heparinized blood. Platelets will adhere to the glass beads, granulocytes will phagocytize the carbonyl iron and red cells will rouleaux by the addition of the methyl cellulose.
4. Rotate conical tubes at 37° C for 30 min.
5. Uncap conical tubes and pull down iron filings with a magnet. Let stand at 37° C for 30 min to allow contaminating cells to settle out.
6. Overlay the lymphocyte rich plasma from each donor onto 10 of the (21) 16 x 100 glass tubes containing 4 ml LSM. Centrifuge tubes at 1800 x g for 10 min with the brake turned off.
7. Harvest each interface and transfer to a second 16 x 100 glass tube. Fill with McCoy's. Mix well and centrifuge at 1300 x g for 10 min. This first wash step removes any LSM or plasma taken up while harvesting interface.
8. Pour off supernatant and combine all pellets from each donor into one 16 x 100 glass tube. Fill with McCoy's and centrifuge at 800 x g for 5 min.
9. Pour off supernatant and Lympho-Kwik™ cell pellets. All cell pellets are Lympho-Kwik™'d to assure removal of any contaminating and non-viable cells.
10. After centrifugation with Lympho-Kwik™, add 2 ml of McCoy's and mix well.
11. In a microcentrifuge tube add equal volumes of cell prep and trypan blue and determine cell viability and concentration microscopically.
12. While counting, centrifuge glass tubes with remaining cell prep at 800 x g for 5 min.
13. Pipet off supernatant and adjust cell concentration to 8 x 10^6/ml in RPMI-azide. Cells are now ready for FITC labeling. (If mixed lymphocytes are used, proceed to Tray Preparation. If separated T and/or B lymphocytes are to be used, proceed first with a separation technique, then to Tray Preparation.)
14. Centrifuge 11 ml of FITC at 7000 x g for 10 min to remove cryoprecipitant formed by the freeze/thaw.
15. To each of 10 microcentrifuge tubes, add 16 drops of cell prep and 5 drops of FITC.
16. Rotate tubes at 37° C for 20 min.
17. Centrifuge 6 microcentrifuge tubes of human serum at 7000 x g for 5 min and transfer to a new tube.
18. Remove tubes from 37° C incubator and wash twice with RPMI-azide. Centrifuge at 2000 x g for 1 min.
19. Pipet off supernatant and add 8 drops of RPMI-azide to one tube. Combine all pellets from each donor, overlay onto human serum and centrifuge at 1000 x g for 5 min. Any free FITC will bind to immunoglobulin in the serum.
20. Pipet off supernatant and wash once with RPMI-azide. Centrifuge at 2000 x g for 1 min.

Tray Preparation

This technique describes the placement of 6 different cells per tray. Modifications can be made as to numbers of cells per tray, e.g., 30-36 as in commercially prepared trays, but completion of the entire procedure should be accomplished in one day to assure good cell viability.

1. Adjust cell concentration to 8 x 10^6/ml in Freeze Media. Final concentration (4 x 10^6/ml) will be halved after the addition of DMSO-Freeze Media.
2. Check viability and labeling with and without ethidium bromide working solution.
3. Cool the suspension to 4° C. DMSO is toxic at room temperature so all suspensions should be maintained at 4° C.
4. Prepare the DMSO-freeze media by slowly adding DMSO to the Freeze Media. The solution becomes warm with the addition of DMSO so allow it to cool to 4° C before adding it to the cell suspension.
5. Once all reagents and cells are at 4° C, add an equal volume of DMSO-Freeze Media to the cell suspension with constant mixing. Allow to cool to 4° C.
6. Dispense cells immediately onto pre-cooled oiled microtest trays in a cold room, if possible.

Freezing of Lymphocytes

1. Controlled Rate Freezing
   a. Turn on controlled rate freezer and bring chamber to 0° C.
   b. Transfer trays to freezer racks and place in chamber.
   c. Insert into the center of the chamber the sample temperature thermocouple stored in 70% alcohol and bring the temperature of the samples to chamber temperature.
   d. When sample and chamber temperatures have equilibrated, lower the chamber temperature 1°/min to -50° C, then 3° C/min to -95° C.
   e. Quickly transfer trays to vapor phase of liquid nitrogen. Trays can be stored for 6-9 months with good cell viability.
   f. Warm freezer chamber to 24° C before turning off.
2. Non-controlled Rate Freezing
   Trays can be frozen without controlled rate equipment. Viability is occasionally poorer and storage time is shortened.
   a. Place trays in a styrofoam container.
   b. Transfer the container to an ultra low freezer (-70° C) for 24 hrs prior to nitrogen storage. Trays can also be permanently stored at -70° C. Cells in trays maintained at -70° C will remain viable for 2-3 months depending on fluctuations of freezer temperature.

Frozen Bulk into Trays

Laboratories often have cells from donors with rare HLA specificities frozen in bulk aliquots. Lymphocytes can be thawed and refrozen in trays according to the following procedure. Due to additional freeze/thaw of cells, there may be decreased viability. Improved viability can be obtained by pre-treatment with deoxyribonuclease (DNAse).

1. Thaw bulk cells within 2½ min of removal from the freezer.
2. Dispense cells immediately onto microtest trays.
3. Freeze trays as previously described.
4. Make sure cells are dispensed onto trays and refrozen within 30 min of thawing.

Thawing of Trays for Antibody Screening

1. Prepare serum samples for testing.
2. Remove one set of trays (6 trays) and thaw on a warm view box.
3. Immediately upon thawing, add serum to the trays.
4. Perform microcytotoxicity assay.

Interpretation

1. The average number of trays obtained from 50 ml of donor blood is 175 (350 for unlabeled trays).
2. Cell viability is between 90-95% on controlled rate frozen trays stored in vapor phase of liquid nitrogen.

Procedure Notes/Troubleshooting

The following precautions should be taken in order to obtain frozen screening trays with viable cells.

1. Use only **pure** lymphocyte preparations, free of platelet, red cell and granulocyte contamination.
2. Freeze trays the same day as the blood is drawn.
3. Control the freezing rate, either by automated equipment or styrofoam containers.
4. Use slow, dropwise addition of DMSO to reagents and cell suspensions.

5. Once frozen, do not allow sample temperature to rise above -60° C.
6. Store trays in the vapor phase of liquid nitrogen. If stored at -70° C, store in a reliable, rarely opened freezer.

References

1. Bate JF, Sell KW: Preparation of frozen lymphocyte panels in Terasaki trays.In: *Histocompatibility Testing*; PI Terasaki, ed.; Munksgaard, Copenhagen; p 633, 1970.
2. Birkland SA: The influence of different freezing procedures and different cryoprotective agents on the immunological capability of frozen-stored lymphocytes. Cryobiology 13:442, 1976.
3. Crowley JP, Rene A, Valeri CR: The recovery, structure and function of human blood leukocytes after freeze-preservation. Cryobiology 11:395, 1974.
4. Farrant J, Knight SC, Morris GJ: Use of different cooling rates during freezing to separate populations of human peripheral blood lymphocytes. Cryobiology 9:516, 1972.
5. Jewett MAS, Hansen JA, Dupont B: Cryopreservation of lymphocytes. In: *Manual of Clinical Immunology*; NR Rose and H Friedman, eds.; American Society for Microbiology, Washington, DC; p 833, 1976.
6. Nathan P: Freeze-thaw-refreeze cycle to prepare lymphocytes for HLA antibody detection or tissue typing. Cryobiology 11:305, 1974.
7. Rowe AW: Biochemical aspects of cryoprotective agents in freezing and thawing. Cryobiology 3:12, 1966.
8. Sollman PA, Nathan P: An improved method for preparing refrozen lymphocytes on plates for microlymphocytotoxicity studies. Cryobiology 16:118, 1979.
9. Strong DM, Sell KW: Functional properties of cryopreserved lymphocytes. Cryoimmunology 62:81,1976.

Growth of Lymphoblastoid Cell Lines and Clones

Edgar L. Milford and Lisa Ratner

Purpose

This section deals with some of the practical and theoretical considerations which investigators and laboratory technologists face when they are working with long-term cell lines of various origins. There has been increasing use of long term cell lines in immunogenetics for a variety of studies including:

1. As reference reagents in tissue typing.
2. As a source for reference DNA for allotyping.
3. For targets in analysis of molecular epitopes recognized by antibody or T lymphocytes.
4. For investigation of the structure and function of the T cell receptor.
5. For the study of antigen presentation and MHC restriction.
6. For the study of signal transduction by numerous cell surface proteins and receptors.
7. For the production of monoclonal antibodies.
8. For the bioassay of lymphokines and monokines.

This chapter deals primarily with the long term culture of cells of T or B lymphocyte origin. It does not cover the many methods now available to immortalize somatic cells such as SV40 transformation, hybridoma formation by fusion, or infection and transformation with Epstein Barr Virus. Broadly speaking, long term cultures can consist of either “normal” cells or “transformed” cells. While “normal” cells presumably have genomic DNA which is identical to that of similar cells found in a healthy individual, transformed cells have altered DNA content. This can happen spontaneously, as in the case of mutant cell lines, cancer lines, leukemia or lymphoma lines, or the DNA may have been purposely altered by an investigator in order to immortalize a cell, to add a gene, or to delete a gene. These purposeful manipulations are sometimes done in a crude way (for example fusing a myeloma with a B cell to yield a hybridoma which has the immortal properties and antibody producing machinery of the myeloma but the particular immunoglobulin determined by the B cell). Alternatively, specific, well defined genes which induce transformation can be amplified in plasmids or by polymerase chain reaction and can be inserted into a normal cell using electroporation, calcium chloride, lipid vesicles, or retroviruses.

Specimen

While most of the long term cell lines of interest to the immunogenetics community are of human origin, there is increasing interest in the propagation of lines which are of murine origin including the following:

Epstein Barr Virus transformed lymphoblastoid cell lines
Myeloma cell lines
Lymphoma and leukemia lines
Deletion mutants
Site specific mutagenesis mutants
Murine transfectants with expressed human gene insertion
T cell lines
T cell clones
T cell hybridomas
B cell hybridomas

Specimens often arrive in the laboratory in a cryopreserved state from other laboratories or from nonprofit or commercial repositories.

Unacceptable Specimens

Specimens which are contaminated with mycoplasma species or other pathogens which may easily spread in the laboratory or bias experimental results are unacceptable except in exceptional circumstances. Mycoplasma in particular can readily spread and contaminate large numbers of lines.

Instrumentation

In order to culture, quality control, and preserve long term cultures of cell lines it is desirable to have access to the following facilities:

1. Freezers, -80° C
2. Liquid Nitrogen Cryopreservation Storage Systems
3. A large refrigerator, 4° C
4. Humidified, temperature controlled incubator with 5% CO_2
5. Sterile laminar flow culture hoods
6. Work room with positive pressure
7. Liquid scintillation counter
8. Fluorescence microscope
9. Inverted phase microscope
10. Centrifuges

Reagents

1. Medium for Cell Culture
   a. Specific Medium Used For Culture*
   b. Metabolic Supplements*
   c. Metabolic Inhibitors*
   d. 100 U/ml penicillin**
   e. 100 µg/ml streptomycin**
   * There is a wide range of specific culture media which are tailored for the growth of cell lines or which provide selective environments which only permit the growth of cells with particular metabolic characteristics because they are toxic to cells without those characteristics.
   ** Routine use of antibiotics should not be necessary if strict sterile technique is used. Nevertheless, in situations when a critical culture is being done, presence of these antibiotics may eliminate low grade bacterial contamination. These antibiotics do not prevent the most insidious problem of mycoplasma contamination.
2. Freezing Solution A
   a. 20% (by volume) Normal Human Serum (AB male) (non-cytotoxic)
   b. 10% Filtered Dimethylsulfoxide (DMSO)
   c. 70% RPMI 1640 Medium
3. Freezing Solution B
   a. 20% [of frozen 1% stock Bovine Serum Albumin (BSA) in RPMI 1640, pH 7.4, 0.2 µ filtered]
   b. 10% Filtered Dimethylsulfoxide (DMSO)
   c. 70% RPMI 1640 Medium
4. DNAse Stock Solution (Sigma D0876, 500 Kunitz units/mg)
   a. 13.3 mg/ml in saline
   Filtered (0.2 m). Store at -80° C in small aliquots
5. Fetal Bovine Serum (FBS)
   Heat inactivate at 56° C for 30 min. Sterile aliquots stored at -80° C.
   Note: Must be mycoplasma-free.
6. Bisbenzamide fluorochrome stain stock, 5 mg
   a. Bisbenzamide fluorochrome stain (Hoechst N 332578, Cal Biochem)
   b. Hanks Balanced Salt Solution (HBSS) 1X without Na_2HCO_3 100 ml
   c. Thimersol (merthiolate, Sigma) 10 mg
   Mix thoroughly, using a magnetic stirrer, for 30-45 min at room temperature (RT). Stain is heat and light sensitive. Store concentrate in an amber colored bottle wrapped completely in aluminum foil, in the dark, at -4° C. Discard when contamination or deterioration occurs. Do not filter.
7. Bisbenzamide Working Solution
   a. Bisbenzamide stock solution 1.0 ml
   b. HBSS without Na_2HCO_3 or dye 100 ml
   Prepare in ginger bottle. Mix thoroughly for 20-30 min at RT, using a magnetic stirrer. Optimal fluorescence may range from 0.05-0.5 µSg/ml.
8. Citric acid disodium phosphate buffer for mounting fluid
   a. Citric Acid 22.5 ml
   b. 0.2M disodium phosphate 27.8 ml
   c. Glycerol 50.0 ml
   Adjust pH to 5.5 (check periodically). Store at 2-8° C.
9. Fixative
   a. Absolute methanol 3 parts
   b. Glacial Acetic Acid 1 part
10. Mitogenic Lectin Stocks
    a. Concanavalin A (Con A) 1 mg/ml Stock or
    b. Phytohemagglutinin (PHA) 1 mg/ml Stock

Lectins should be carefully weighed and brought to concentration in sterile distilled water. Stock solutions should be centrifuged at 7,000 rpm for 30 min to remove particulates and aggregates, then filtered through a 0.2 μ filter prior to freezing at -80° C in convenient aliquots.

Procedure

The propagation of long term cell lines and testing these lines for contamination with mycoplasma is a technical art. No rigid guidelines can be stated since each line, clone, and subclone which is propagated will have distinctive requirements for optimal growth. Recognition of some of the major variables which affect one's ability to successfully propagate and preserve cells can aid the technologist in what is often a labor-intensive enterprise. While some cell lines are extraordinarily robust and grow readily and indefinitely in plain RPMI 1640 medium without protein or supplements, other lines and clones require specific metabolic supplements, selected lots of serum protein, or stimulation with a combination of lymphokines, monokines or antigen at specified intervals. Even under optimal conditions and with maximal vigilance, some lines and clones experience programmed senescence and go into a phase of inexorable decline from which there is no recovery.

General Requirements for Growth

All cells grow more vigorously in the presence of a source of protein than in medium without protein. More accurately, cells maintain their growth characteristics better in the presence of autologous serum than without a serum source. It is thought that a small amount of a protein such as albumin is needed to stabilize cell membranes and maintain conformation of important cell surface proteins (receptors) which play a role in the uptake of metabolites needed for growth. In addition, other components of serum enhance cell growth and produce better viability. These components include transferrin, insulin and selenium, all of which may be added to medium to improve rate of cell proliferation and viability.

It is possible to grow most cells for limited periods of time, and many cells for long periods of time, in defined serum-free nutrient medium containing essential electrolytes, a source of sugar, and essential amino acids (RPMI-1640 is one such commercial preparation). The medium can be supplemented with 0.25-0.5% bovine or human albumin, transferrin, insulin, and selenium. Glutamine is an amino acid which degrades rapidly and may need to be added to the medium to improve cell growth. This type of serum-free medium can be used when the investigator is interested in isolating supernatant factors, or measuring lymphokine production from short term cultures.

Hybridoma Growth

T cell and B cell hybridomas are generally produced as follows:

1. Insure that the malignant fusion partner is deficient in the "exogenous" purine synthesis pathway by growing it in 8-Azaguaninine. This agent is taken up by cells which have an intact exogenous purine synthesis pathway, and those cells are eliminated, leaving only those which are deficient.
2. Physically fuse two cell populations with each other with polyethylene glycol. One population is a malignant "immortal" line. The other is a normal T or B cell population which has the characteristics one wishes to conserve in the "hybridoma" product of the fusion between the two cell types.
3. Isolate the hybridomas from the malignant parent cells by pharmacologic selection. The parent is usually a special line which has been selected for deficiency of an enzyme necessary for synthesis of purines from exogenous precursors. After the fusion is effected, one cultures the hybridoma in medium containing aminopterin, an agent which poisons the endogenous purine synthesis pathway. Since only the "normal" partner can contribute the exogenous synthesis pathway, only cells which have fused will continue to grow. Unfused normal partner cells usually die on their own because they have not been immortalized. The selection medium (called HAT) also contains hypoxanthine and thymidine which act as substrates for purine synthesis.
4. Grow the hybridomas at limiting dilution so that "clones", or progeny of single hybridoma cells are replicated. This is usually necessary because each primary hybridoma cell will be somewhat different, having different DNA content, and markedly different genotype and phenotype. At this stage it is possible to select the clones one wishes to propagate further on the basis of the clone's characteristics.
5. Cryopreserve aliquots of the primary clones of interest.
6. Subclone the primary clones into subclones. This is done in order to statistically insure that one really has the progeny of one cell.
7. Cryopreserve aliquots of the subclones of interest.
8. Grow hybridoma in "HT" medium, i.e. medium with hypoxanthine and thymidine without aminopterin. Some hybridomas exhibit innate instability and have a high frequency of "reversion" towards the parent tumor genotype (presumably by "kicking out" some of the normal partner cell's genetic material). With this type of line, it is best to continue growing and expanding the hybridoma in HAT medium to maintain continuous selection.

T cell lines and T cell clones

Unlike transformed cell lines and clones, "normal" T cell lines and clones usually require exogenous activation signals to maintain their growth in vitro. Several different approaches can be taken to stimulate the growth of somatic T cell

lines. These approaches involve provision of antigen which can bind to and stimulate the T cell receptor, interleukins which act as ligands for their respective receptors and increase cell proliferation, and "feeder" cells which variably act as a source of cellular kinins, act as antigen presentation agents, or provide an optimal microenvironment for cell growth.

Antigen Stimulation–Cellular Stimulators

Some cell lines are dependent on antigen stimulation to maintain growth and physiological activity (such as cytotoxicity potency). In the case of T cells which are responsive to major histocompatibility alloantigen, the stimulatory signal is usually provided in the form of "stimulator cells". The simulator cells are usually irradiated at 2000-5000 rad or treated with a metabolic inhibitor such as mitomycin in order to prevent the stimulator cells from proliferating themselves. Typically, the number of stimulator cells added to the culture ranges from 10% to 50% of the number of responding T cells in the culture vessel. This depends upon the nature of the stimulator cell population. For example, if the irradiated stimulators are unfractionated mononuclear cells derived from peripheral blood, spleen, or lymph node, the optimal ratio of stimulators to responders is usually 1:1 to 1:3. In contrast, when the stimulator cells are lymphoblastoid cell lines or purified B lymphocytes, stimulator to responder ratios as low as 1:10 can be effective.

Antigen Stimulation–Nominal Antigen

In order for purified T cells to respond to nominal antigen (i.e. antigen protein or peptide in the form of a solution or suspension), the antigen must be presented within the antigen-binding groove of a major histocompatibility molecule to the T cell responders by "antigen presenting cells". Furthermore, larger proteins may need to be "processed" by a cell with "antigen processing" capacity before they can be presented. Therefore simple addition of nominal antigen to a T cell line or clone will usually not result in stimulation and a growth boost. One method for stimulating with nominal antigen is as follows:

1. Plate antigen processing/presenting cells (macrophages, B-cells, B-lymphoblastoid lines) onto a flat bottomed microtiter dish or flask and culture for 12-24 hrs.
2. "Pulse" antigen presenting cells with the nominal antigen at the appropriate concentration for 24 hrs at 37° C in a moisturized 5% CO_2 incubator.
3. Wash the antigen presenting cells so that excess nominal antigen is removed.
4. Add T cell lines or clones to the antigen presenting cells.

Antigen Stimulation–Mitogen

Because T cells contain cell surface glycoproteins which normally serve to receive activation signals through specific ligands, such as antigen-MHC complexes, lymphokines, or cell-bound "adhesion" molecules, it is often possible to mimic the activation signals which these normal stimulatory ligands provide by using a lectin. Lectins are plant proteins which avidly bind to specific sugar residues and therefore to the glycosylated residues of many cell receptors. Stimulatory signals transduced by binding of lectins often result in nonspecific mitogenic responses. Phytohemagglutinin (PHA) and Concanavalin A (Con A) have been extensively used to induce T cell proliferation in vitro.

1. Add PHA to cell culture at 2 µg/ml net concentration or Con A at 10 mg/ml net concentration.
2. When cells have responded with log phase growth, decant and change medium to remove excess mitogen.

Stimulation with Crosslinked Anti-CD3

Stimulation of the T cell receptor directly and specifically with monoclonal antibody against its CD3 component is an effective way of inducing mitogenesis in T cells. In order to provide the proper signal, the antibody-receptor complexes need to be crosslinked either by a second antibody which binds to the anti-CD3 or by first binding the anti-CD3 to a solid support such as the surface of a plastic culture plate or plastic beads. Depending on the nature of the cells being propagated it may be necessary to add feeder cells which provide complementary lymphokine.

Lymphokine Requirements

Unlike transformed cell lines and clones, normal T cell lines and clones usually require exogenous interleukins to maintain their growth in vitro. Interleukin 2 (IL-2) appears to be the most important of these lymphokines, since supplementation of medium with recombinant interleukin-2 is usually sufficient to maintain growth. It must be appreciated, however, that some lines appear to depend on other interleukins such as IL-4 and IL-6. Yet other lines grow best when the medium is supplemented with 5-20% of a crude supernatant from normal lymphocytes cultured for 24 hrs in 10 mg/ml of Concanavalin A or 2 µg/ml of PHA.

Feeder Cells

Even in the presence of optimal formulations of medium, cofactors, protein, lymphokines, and stimulation of the TCR with antigen or anti-CD3 antibody, it is often necessary to provide live feeder cells to cell cultures, in particular when subcloning or culturing a very small number of cells is needed. The best human feeder cells appear to be peripheral blood lymphocytes which have been irradiated at 1000 rad to prevent them from dividing. It is generally not necessary to have feeders in cultures which are growing well at moderate to high cell density or in cultures of transformed cells. The mech-

anism by which feeder cells work is not known. To some degree the feeder cells are a source of interleukins, lymphokines and monokines which act as growth factors. Even if one adds a sufficient amount of growth factors (recombinant or derived from the supernatant of a mitogen-stimulated 2 day culture of lymphocytes), some cell lines require the additional presence of feeder cells, which suggests a role for cell-cell contact in the requirement. Feeder cells are typically plated in fresh medium and allowed to remain in the culture vessel for 12-24 hrs prior to adding the cells which need to be propagated.

Cloning and Subcloning

Cloning and subcloning are techniques for obtaining a population of cells which are all the progeny of a single cell. Most commonly this is accomplished by the "limiting dilution" technique. The principle of this method is that one dilutes a mixed population of cells sufficiently so that when the suspension is plated into multiple microtiter culture wells there are usually no more than 1 cell per well. In order to insure that no more than 1 cell is in the average well, a dilution is chosen which actually results in most wells having no cells and a few having 1 cell. In fact, significantly fewer wells ultimately have cell growth than would be calculated by the dilution in which they were plated because there is variable loss in viability when cells are grown at such extreme dilution. This can be obviated somewhat by pre-plating irradiated syngeneic feeder cells; however some loss always results in a "cloning efficiency" of considerably less than 100%. Cloning efficiencies range from close to 90% with transformed cell lines to under 30% with some T cell clones. A typical protocol for cloning is as follows:

1. Prepare a monodispersed cell suspension by washing cells in medium, counting cells under a phase microscope in a counting chamber, and vigorously dispersing cells by passing through a sterile syringe fitted with a #22 needle. It is extremely important to insure a monodispersed cell suspension. Cell clumps, duplets and multiplets will defeat the purpose of dilutional cloning and result in one well containing the progeny of more than one initial cell.
2. Dilute cells sufficiently so that less than 1 cell in every 10 wells will result in a proliferating population of cells when finally plated. Unfortunately it is rarely known what the cloning efficiency will be ahead of time so it is wise to follow the procedure in "3" below.
3. If cloning efficiency is not known, set up multiple microtiter plates with serial dilutions of cells (one dilution for each series of plates) over a range of five 2-fold dilutions (from 5 cells/well to 0.25 cells/well). The dilution which results in an average of 1 positive well out of 10 should be used as the basis for future clonings.
4. If feeder cells are used they should be plated 12-24 hrs prior to adding the cells to be cloned. Feeders are used at a density of 5-10 x 10^3/well.
5. When positive wells are in log phase growth, resuspend individual positive wells and distribute over 6 new wells with added feeders and medium. When 6 wells have grown sufficiently by microscopic inspection, cryopreserve contents of 3 wells, and transfer pooled contents of other three wells to the well of a 24 well flat bottomed microtiter plate.

Confirmation of Identity

It is important to perform regular identity checks on cells which are grown long term. Specific isolates can become cross-contaminated with cells of other origins, and hybridomas and transfectants can spontaneously mutate, changing genotype or phenotype. Although strict adherence to good laboratory practice should avoid the former problem, the latter phenomena cannot be prevented, and only extensive programs of cryopreservation of early confirmed samples of lines can insure availability of original isolates. Without doing extensive DNA fingerprinting, or indeed total genomic sequencing, it is impossible to verify that any particular sample of a named cell line or clone has not had any alteration in the genomic DNA. It is, however, possible to carefully monitor for gross cross-contamination, change in karyotype, and changes in important phenotypes that are characteristic of the cell in question. The following are examples of variables which can be followed. These techniques are not included in this chapter, but most are detailed elsewhere in this volume.

1. Species Identity
   a) Karyotype specimen (cytogenetics laboratory)
   b) Use species specific monoclonal antibodies to characterize by fluorescence cytometry.
2. Differentiation antigen phenotype (flow cytometry with antibodies against CD4, CD8, CD3, V-beta MoAbs, etc).
3. Cell Surface Allotype of Cells
   a) HLA typing (microcytotoxicity)
   b) Isoelectric focusing of class I
   c) 2-D gel electrophoresis of class II
4. DNA Genotype
   a) Southern Blot with probes against HLA
   b) Southern Blot against multiple unlinked loci which exhibit allelic variants (repeat sequence probes)
   c) Polymerase chain reaction amplification of specific loci and dot blotting with informative sequence specific oligonucleotides
5. Function
   a) Secretion of characteristic lymphokines (IL-2, etc.)

   b) Detection of T-cell receptor dependent responses
      1. Proliferation in response to specific antigen
      2. Lymphokine secretion on exposure to antigen
      3. Cytotoxic response against specific cells
6. Morphology
   a) Size and shape of cells in log growth under phase contrast microscopy
   b) Lymphoid, blastoid, stellate, epithelioid
   c) Appearance with Wright's-Giemsa stain
7. Growth Habit
   a) Rate of growth (doubling time) in log phase
   b) Lymphokine dependence
   c) Radiation sensitivity
   d) Monolayer formation (adhesion to culture vessels)
   e) Autoadhesion (clumping vs. monodispersed)

Infection

The inadvertent introduction of unwanted organisms into long term cultures can be avoided by proper technical procedures and maintenance of equipment. The most frequent culprits are mycoplasma, bacteria, yeasts, fungi, and viruses. While bacteria and yeasts quickly make themselves apparent to the technologist, mycoplasma and viral species present a more serious problem since they can be present in a culture for some time without declaring themselves, and spread throughout the laboratory unless actively monitored.

Procedure Notes

Prevention of Infection

A. Set-up of Work Area
   1. The work area should preferably be in a cul-de-sac of the laboratory, without extensive through-traffic, and dedicated to sterile work.
   2. The air supply should be as clean as possible. In particular, primary dust filters in the air conditioning system should be supplemented by secondary fine mesh filters.
   3. Air conditioning filters should be cleaned frequently to prevent accumulation and dispersal of fungus on the near side of the filters into the work area.
   4. The humidification system should be inspected by the environmental safety division of your institution on a regular basis to determine that microorganisms are not growing on, and atomized from, the permanently water-saturated surfaces.
   5. Vertical laminar flow hoods with HEPA filters and a device for "flaming" the mouth of culture vessels should be available. Some fire codes prohibit open flame devices in hoods, however there are alternative devices available.
   6. The services of an exterminator should be used to assure that vermin are eliminated from the work space and adjacent structures. Potent, safe, and effective agents are available for placement in industrial laboratory spaces.
   7. The working surfaces, walls, floors, and preferably the ceilings should be of a smooth nonporous substance which is easily cleaned and which resists accumulation of dust.
   8. The floors and working surfaces should be cleaned daily with a germicidal detergent solution.

B. Incubator Use
   1. There should be dedicated incubators for long term culture of cell lines.
   2. There should be at least one back-up incubator so that incubators can be thoroughly cleaned at least once per month.
   3. Incubators should be cleaned with a nonabrasive household detergent, then rinsed with distilled water. For a moisturized incubator, fill two shallow aluminum freezer pans with water and put several copper pennies (pretreated with hydrochloric acid until they are shiny) in each. We have found that this is as effective as antifungal agents such as mycostatin in inhibition of fungal growth.

C. Sterile Laminar Flow Hood Use
   1. Keep the sterile hood free of all objects when not in use. Objects stored in sterile hoods act as repositories from which organisms spread to your cultures.
   2. Use germicidal ultraviolet light when not actively using the hood. Do not be comforted by your ultraviolet light if you use your hood as a storage closet. Bacteria can hide from UV light on the distal sides of any object.
   3. Prior to use of a sterile hood, spray the work surface with a mist of 70% isopropyl alcohol (using a plant spray bottle), and wipe clean with a towel after soaking for 5 min.

D. Culture Medium Precautions

   The single most important contributor to infected cultures is grossly contaminated culture medium.
   1. All medium, serum, lymphokines, mitogens, and growth factors should be available in demonstrably sterile sealed aliquots.

2. Aliquots should preferably be used at one culture session, or if not within 2 days of opening. Once a bottle is opened there is a finite chance that it has become contaminated. The longer a slightly contaminated aliquot sits (even at 4° C) the more serious the consequences of a few stray organisms.
3. Frozen sterile aliquots which need to be thawed should only be thawed in a water bath if that bath is aseptic. All too often water baths are teeming microbial soups which coat the entire outside of your aliquot vessel with countless organisms which are then transferred to your hood. Non-volatile aliquots should simply be thawed by leaving closed at RT.
4. All medium and additive containers should be wiped with new cotton gauze dampened in 70% isopropyl alcohol prior to use.

E. Technologist Operation Procedure
1. Wash your hands prior to culture session. Wear surgical gloves. If gloves are powdered, wipe the outside with gauze dampened in isopropyl alcohol.
2. Wear a clean lab coat and surgical mask.
3. Wear a surgical cap if you have long hair.
4. Do not talk at the hood
5. Remove watches and dangling jewelry, etc.

F. Use of Pipettes
1. It is impossible to reliably and consistently remove volumetric pipettes from the plastic containers in which they come in a sterile fashion over multiple culture sessions. Pipettes used in this manner will become contaminated with organisms from the outside of the container or your fingers.
2. Purchase large metal canisters made for holding pipettes, and resterilize the pipettes in an autoclave with a standard protocol, or using dry heat (250° C for 12 hrs). Use smaller metal or glass canisters for Pasteur pipettes, and sterilize them with dry heat.
3. Pasteur pipettes should be purchased with cotton plugs in the wide end. This minimizes droplet contamination from the bulb or vacuum pipettor, or worse, unrecognized cross-contamination of cell lines themselves.

Biohazard Precautions and Prevention

All long term cell lines, whether of malignant origin, normal, or transformed with viruses must be considered to be biohazards. This is particularly true of human lines. 25-30% of Epstein-Barr virus-transformed B lymphoblastoid cell lines are known to secrete active EB virus, and therefore can be infectious. Some T cell lines and malignant lines have either been purposely transformed with a retrovirus such as HTLV1 or come from an individual who may be infected. Some of the strains of mycoplasma which contaminate cultures are also infectious pathogens for humans and can cause pneumonia.

1. Avoid aerosolization of cultures.
2. Always use vertical (isolation) and not horizontal laminar flow hoods.
3. Dispose of pipettes into an appropriately labeled container or blood receptacle after decontaminating, as dictated by relevant regulations which apply to biohazards.
4. Household bleach, diluted 1:5 with tap water is a commonly used decontaminant solution.

Cryopreservation

Cell Freezing Technique

Virtually any cell line which grows well can be cryopreserved. The viability of cryopreserved cells is critically related to the condition of the culture at the time it is frozen.

1. Cells should be frozen during early logarithmic growth phase when there is greater than 90% viability and little cell debris in the preparation.
2. Freezing Solution A or B stocks (see above) can be used for cryopreservation of most cell lines. Fetal calf serum (FCS) can be used as a substitute for normal human serum in most cases, but some lots of FCS are toxic to individual cell lines. For Epstein-Barr Virus transformed lymphoblastoid cell lines it is best to use 40% FCS, 10% DMSO and 50% RPMI as the stock freezing solution.
3. To freeze cells slowly add an equal volume of 4° C freezing solution A or B (or alternative freezing solution above) dropwise, to a 4° C suspension of cells in RPMI 1640 medium with constant gentle mixing.
4. Immediately pipette into plastic freezing vials and place upright in tube racks in a -80° C freezer. It is advisable to rest the tube rack in an open styrofoam box within the freezer to promote an even rate of cooling.
5. Use a chest freezer if possible, or if not, use the bottom shelf of an upright freezer. This will minimize the temperature fluctuation caused by individuals opening the doors.
6. Transfer cells to containers in a liquid nitrogen cryopreservation vessel 24-48 hrs afterward if long term storage is desired. Very efficient vessels are now available which have vacuum sealed walls, low heat absorption, moderately high sample capacity, and which need to be filled only once every few months.
7. It is not necessary to differentiate between the “liquid phase” and the “vapor phase” of liquid nitrogen. There will be no difference in average viability.
8. Most transformed cell lines maintain excellent viability, even at -80° C for periods of up to 1 year. T cell clones and lines should be stored at liquid nitrogen temperatures.

9. EBV transformed lines, T cell leukemias and lymphomas and hybridomas are optimally frozen at densities of 2-5 x 10^6/ml. T cell lines and clones can be frozen at up to 10 x 10^6/ml. Beyond a density of 10 x 10^6/ml, the DNA released by dead cells upon thawing is sufficient to form a mesh or "clot" which can entrap live cells.

Cell Thawing

1. It is important to inspect all tubes immediately on removing from the liquid nitrogen to insure that there is not any liquid nitrogen inside the tube. Rapid evaporation of the liquid nitrogen can make tubes explode and cause serious damage even if the tube is plastic. It is advisable to twist the top of the tube slightly so that expanding vapor can escape. Particular care must be taken with the older glass vials, which should no longer be used.
2. Cells in standard 1.5-2 ml cryopreservation tubes are best thawed by rolling the vial in the palm of your hand until most of the ice is gone.
3. Transfer cell suspension to a 10 ml plastic tube, bring to 10 ml in RPMI 1640, and centrifuge at 1200 rpm for 5 min. Carefully decant medium, wash one more time, add 5 ml of RPMI with 5% fetal calf or normal human serum, and resuspend cells.
4. Cells can be freed of nuclear debris and aggregated DNA by a brief incubation with DNAse. Use 0.5 ml of the Stock DNAse solution (see above) added to the cell suspension, and incubated at 37° C for 10 min. Wash twice in RPMI with 5% protein. If cells are to be used for making a fresh DNA preparation, it is wise to wash extensively or to culture cells for 24-48 hrs before making the preparation.

Propagation of LCLs

Because EBV-lymphoblastoid cell lines have been transformed, they do not appear to depend upon exogenous lymphokines for their growth. They are the easiest of cells to grow. The most critical factor in growing these cells is daily microscopic inspection of each set of cultures to determine when the culture should be "fed" or "split". The following are guidelines for growing LCLs.

1. When cells are thawed, start culture in vessels with a small surface area (either 72 well or 0.2 ml culture plates or slightly larger 2 ml capacity wells).
2. Inspect cultures daily under inverted phase microscope to assess viability and rate of growth of cultures. Use a consistent scoring system and keep record of the two variables on each culture, as cell lines tend to maintain growth characteristics.
3. When cultures are growing (i.e. >50% of the surface area of the well is covered with live cells) and there is a barely perceptible decrease in the pH of the medium by indicator dye, split the cultures 1:2 to 1:4 and add more fresh medium to each of the new wells, or transfer directly into a small culture flask as below.
4. When the split cultures are at a similar stage of growth as in step 3, split half of the wells 1:2 and pool the other half into a larger culture vessel such as a 45 ml plastic culture flask. Do not put more than 15 ml of medium into the 45 ml culture flasks if you intend to inspect the cultures, as it will be impossible to turn the flask on the side and inspect under phase microscopy without liquid touching the neck and flask cap. When inspecting cultures, make sure flasks are allowed to settle on their sides long enough for cells to float to the side near the microscope objective. Live cells will be the last to settle.
5. Cultures should always be fed when the medium starts to develop a distinct yellow tint, indicating acid pH. There are two ways of feeding: adding fresh medium to a partially filled flask, and changing the medium. We favor changing the medium by flaming the vessel mouth, quickly decanting approximately 90% of the supernatant (without resuspending the cells!), flaming again, and then adding fresh medium which has been allowed to warm to 22-37° C.
6. Cultures must be split when growth becomes confluent. Even if the cells appear to be healthy, it is then impossible to accurately assess the viability and rate of growth. Split cultures by gently swirling the vessel to resuspend cells. Then divide the volume approximately equally into identical new vessels. In some cases, when one wants only to maintain a culture, simply discard 80% of the suspension (including the cells), then refill the original vessel with medium.
7. When you are comfortable with the growth of your cell line and need not inspect it frequently, you may want to fill the flasks with medium and culture in an upright position. While this prevents day to day microscopic inspection it permits longer continuous growth without changing medium.

Control for Mycoplasma Infection

The threat of mycoplasma infection in cell cultures is the bane of investigators using long term cell lines. The longer a culture is growing, and the more lines simultaneously in use in the lab, the better chance there is for mycoplasma contamination. One of the greatest problems is that mycoplasma can remain undetected for long periods of time. This section will give a basic outline to the problems, causes and effects of mycoplasma, and a description of preventative measures that should be applied when working with cell cultures.

Quality Control of New Specimens

1. Obtain documentation of mycoplasma testing on all new cell lines which come into the laboratory for propagation or use in the incubators or hoods.

2. Test all new cell lines for mycoplasma which have not come with documentation of mycoplasma free status.

Monitoring After Mycoplasma Has Been Identified

When there is a problem with identified mycoplasma contamination in the laboratory the following steps should be taken to identify the sources:

1. Make sure that you test your lines regularly for mycoplasma. For the most accurate results, both the direct and indirect methods (described below) should be utilized. Ultimately, there is no absolute guarantee against mycoplasma infection. It is advisable to try to isolate the cause of infection at first. If the contamination is limited to one or two cultures, chances are that the source of infection is from the person handling the cultures. The importance of good sterile technique cannot be emphasized enough.
2. Check the medium and serum supply. Fetal calf serum should be heat inactivated at 56° C for 45 min, centrifuged to remove large particulates, and immediately pipetted into convenient aliquots which will be used over time within 48 hrs of opening. Fresh bottles of sterile medium are generally used. If the volume of work does not permit use of a 500 ml bottle of medium within 48 hrs, use 100 ml bottles instead. Both FCS and medium as shipped should be completely sterile. If there is contamination in more than one aliquot check with vendor.
3. Clean the hood and check filters.
4. Shut down incubator, wash with a 20% bleach solution, and autoclave all of the racks.
5. Wash the floors and walls of culture room with antiseptic solution.
6. Monitor room and incubator for general level of airborne contaminants (not necessarily mycoplasma) by setting out agar plates for 1, 2, 3, 5, and 10 min. Compare with baseline results when contamination of cultures was not a problem.

Testing Cultures for Mycoplasma

A test for mycoplasma should be repeated each time a cell line is thawed and substantially expanded by culture for cryopreservation into a new stock. There are two methods for testing: The direct method and the indirect method. Both of these methods have limitations and all these limitations should be taken into consideration when testing for contamination. The limitations of these indirect assays are:

1. Radioactive materials must be used with DNA probes.
2. Some indirect methods are not as sensitive for detecting minute quantities of mycoplasma contamination as are cultures. One cannot identify the different types of mycoplasma, however the commercial kits now available are more sensitive.

Advantages of the indirect methods are:

1. Indirect methods are a speedy way of detecting substantial mycoplasma contamination.
2. One can detect M. hyorhinus strain, which is a major source of contamination and difficult to culture.

Direct Microbiological Agar Culture

Cultures take about 3 weeks. It is essential that the culture be grown in antibiotic-free medium. Using the direct method, very small numbers of mycoplasma organisms can be detected, identified, and isolated for subtyping. This method is much more sensitive than the indirect method. Most strains of mycoplasma hyorhinus cannot be cultured, however, so it is best to use indirect and direct methods together to insure accurate results.

1. Samples of the cell suspension are inoculated in aerobic broth at 37° C and other aliquots are plated with anaerobic agar plates. Turbidity and pH are observed as signs of contamination over 14 days. Anaerobic plates are examined weekly for 3 weeks for mycoplasma colony formation. In agar, mycoplasma colonies often appear like "fried eggs."[2] Choices of medium components for culturing mycoplasma vary from lab to lab. Standardizing culture procedures can influence the successful growth and isolation of mycoplasma, so pretesting of media with defined strains is essential. The medium will depend on which mycoplasmas are being examined. For example, 2% arginine stimulates growth,whereas <0.5% arginine inhibits growth of certain strains. Too much agar can inhibit colony formation. Because of the complexity of culture for screening of mycoplasma, it is a task best left to a professional microbiology laboratory that specializes in the identification of this organism. The following should be kept in mind when sending cultures for mycoplasma:
   a) Fresh aliquots of growing culture should be used, including the cells.
   b) A large proportion of the hyorhinus type of mycoplasma, a major source of mycoplasma contamination, cannot readily be detected in culture.
   c) Culture routinely takes 3 weeks, so results are not readily available.

Indirect Identification of Mycoplasma Contamination

Indirect identification of mycoplasma can be achieved by directly staining the organism and inspecting under a microscope, or by binding of specific probes to the DNA of the organism and detecting positive binding using a radioactive tracer.

Hoechst Staining Technique

1. Cell cultures to be tested for mycoplasma infection are grown on a cover slip or microscope slide.
2. Plate approximately 5 x 10^4 cells in 0.5 ml onto a 22 x 22 mm cover slip in a 60 x 15 mm Petri dish. Place in CO_2 incubator (5% CO_2) for 4 hrs to allow complete cell attachment onto the cover slip.
3. After 4 hrs, gently add 3 ml medium and return to CO_2 incubator for 24-48 hrs.
4. Label 4 slides "positive" and 4 slides "negative".
5. At 24-48 hrs, remove from incubator and add about 10 drops of fixative directly to dish, making sure that drop placement is beside the cover slip. This is done without decanting medium or disturbing cells.
6. Two min later, suction the entire solution off, and add 3 ml of fixative. Allow 5-10 min of fixation.
7. Suction off the fixative until no liquid can be seen around the cover slip. Remove the cover slip. For ease of removal, squeeze the plate in the palm of your hand and lift up the cover slip with forceps. Place the cover slip against the inside wall of the plate. From this step on, be sure to keep track of which side the cells are on!
8. Dry on a slide warmer or in an oven at 55-60° C for 10-15 min.
9. Stain completely dried cell preparation by adding 3-4 drops of working solution (0.5 mg/ml of H-stain) to the cell surface and invert cover slip onto a clean microscope slide (cell surface now faces down and is immersed in staining solution trapped between cover slip and glass slide). Do not press out excess stain. Leave preparation in this position for 10 min.
10. Mount cover slip onto a clean microscope glass slide with the cell surface down using the citric acid mounting fluid. Drain off excess mounting medium by placing blotting paper along the edge of the cover slip.
11. Examine preparation using fluorescence microscope. When looking through the microscope the nuclear DNA of the cell culture is easily visible and appears as large (15-20 m) spherical bodies. The mycoplasma DNA appears in contrast as small numerous fluorescing particles (0.5-1 m). Large thin filamentous strands of mycoplasma DNA may also be evident.[2]

Indirect DNA Probe Techniques

New, sensitive, and simple kits for the detection of mycoplasma, which rely on DNA hybridization techniques, have become available. DNA is composed of 2 strands, each strand being complementary to the other. The strands can be reversibly separated and reannealed to their original complementary strand or to any other complementary sequence present. Although RNA is single stranded, it shares this ability to bind complementary nucleic acid sequences. The mycoplasma detection kits consist of small tritium labeled DNA probes which are complementary to known specific sequences of mycoplasma RNA. Such a probe is added to an aliquot of supernatant from the cell culture to be tested. mycoplasma RNA present in this cell culture supernatant hybridizes to the labeled complementary specific DNA probe. If mycoplasma is present, double stranded DNA-RNA hybrids are formed and are then detected in a scintillation counter. Each kit has its own specific methodology.

References

1. Barile MF, Mycoplasma contamination of cell cultures: Mycoplasma-virus-cell culture interactions. In: *Contamination in Tissue Culture*; J Fogh, ed.; Academic Press, New York, p 132, 1973.
2. Barile MF., In: *Cell Culture and Its Applications*; R Acton, JD Lynn, eds.; Academic Press, New York, p 291, 1975.
3. Chen TR, Utilization of fluorescent Hoechst stain to effectively detect mycoplasma contamination. Vitro 10:390, 1974.
4. McGarrity GJ, Detection of mycoplasma infection of cell cultures. In: *Advances in Cell Culture*. Vol. 2, 1982.

Preparation of B Cell Lines

Paul J. Martin

Principle/Purpose

Immortalized human B-lymphoblastoid cell lines (BLCL) infected with Epstein-Barr virus (EBV) represent extremely useful and convenient reagents for serological, biochemical, functional, and molecular biological studies of major histocompatibility complex (MHC) molecules and genes. Such cell lines can be maintained indefinitely, expanded to numbers limited only by the availability of culture medium and incubator space, or cryopreserved and readily reestablished in culture when needed.

EBV is ubiquitous in the human population and nearly all adults are EBV-seropositive. Under certain conditions, BLCL can be generated "spontaneously" in cultures of peripheral blood cells from EBV-seropositive individuals, reflecting the transforming activity of virus persisting in a small number of B cells.[5] More commonly, BLCL are established by deliberate infection of peripheral blood B cells with exogenous EBV. Human BLCL produce little, if any, infectious EBV, but large quantities of the virus are produced by EBV-infected marmoset B cells.[4] When peripheral blood cells of seropositive individuals are infected with EBV in vitro, foci of proliferating transformed B cells can be observed during the first 7-14 days, but this is followed by a regression of growth caused by cytotoxic T memory cells that recognize virus-encoded cell surface antigens.[6] Destruction of the nascent BLCL can be avoided by removing T cells before establishing the cultures,[7] or by inactivating T cells with cyclosporine1, CD3 antibody,[8] or with an antibody that blocks the binding of IL-2.[8]

Specimen

Peripheral blood mononuclear cells (may be cryopreserved). Cells must be viable.

Reagents and Supplies

1. Cyclosporine – 100X stock
   Pharmaceutical cyclosporine (Sandoz) is supplied at 50 mg/ml in 5 ml ampules.
   Dilute cyclosporine 1:250 in medium to provide 100X stock. Store at 4° C.
2. RPMI 1640 medium
3. Fetal calf serum (FCS) or fetal bovine serum (FBS)
   FCS or FBS must be mycoplasma-free and tested for cytotoxicity against lymphocytes
4. Ficoll-Hypaque

Instrumentation/Special Equipment

1. Laminar flow hood
2. CO_2 incubator
3. Centrifuge

Calibration

Incubator must maintain a humidified atmosphere of 5% CO_2

Quality Control

1. BLCL in long term culture can become contaminated with bacteria, fungi and mycoplasma. Bacterial and fungal contaminations are readily apparent, usually indicated by a drastic pH change and increased turbidity of the medium. Mycoplasma infections are insidious but can be detected by a variety of methods. Contamination of B95-8 with certain mycoplasma strains will prevent EBV transformation. Maintenance of cultures in antibiotic-free medium may reduce the incidence of unrecognized mycoplasma contamination since poor culture technique will sometimes cause a concurrent bacterial or fungal contamination which can be recognized readily. When contamination occurs, the culture must be destroyed and a new culture started from cryopreserved cells.
2. Cross-contamination between human cell lines and with xenogeneic cells has been well documented.[2] Cytogenetic analysis, HLA and other surface markers, polymorphic enzyme markers, and DNA restriction fragment length polymorphisms should be monitored in order to verify the identity of cell lines.[3]

Procedure

Preparation of Culture Supernatant Containing EBV

1. The B95-8 EBV-producer cell line can be obtained from the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852 (catalogue number CRL 1612). This cell line is readily maintained at

0.3-2.0 x 10^6 cells/ml with twice weekly feeding in RPMI 1640 medium containing 8-12% fetal calf serum or iron-supplemented calf serum (Hyclone, Logan, UT) and 2 mM added glutamine.
2. Grow cells at 37° C in a humidified atmosphere containing 5% CO_2. The B95-8 cell line should be handled with P2 (BL-2) precautions.
3. Prepare virus stock by seeding cells at 0.3 x 10^6/ml in fresh medium in a large (75 or 200 cm^2) tissue culture flask and leaving the culture undisturbed for 6-8 days. Virus production may be enhanced by culturing cells at 32-34° C.
4. Recover culture supernatant by centrifuging cells at 400 x g for 10 min and filtering through a 0.45 or 0.22 µ vacuum filter (Nalgene, Rochester, NY).
5. Store small aliquots (1-5 ml) at -70° C to -90° C.

Under these conditions, transforming activity of the virus can be maintained for at least 12 months.

Peripheral Blood Mononuclear Cells

1. Isolate mononuclear cells under aseptic conditions from 10-30 ml of heparinized blood (10 u of heparin/ml blood) by centrifugation over a FH gradient (S.G. 1.077) at 900 x g for 20 min.
2. Wash interface cells three times in PBS and resuspend in medium at 2 x 10^6 cells/ml.
3. Use bovine serum for all cultures; human serum contains EBV-neutralizing antibodies.
4. If cyclosporine, anti-CD3 or anti-CD25 antibody is not used to facilitate transformation, then deplete T cells by any suitable procedure such as E-rosetting, nylon wool column filtration or complement-mediated lysis (see the chapter on lymphocyte isolation).
5. Dispense cells (2 x 10^6/ml) in a vessel of suitable size and add an equal volume of thawed virus stock. Cultures can be established in 0.2 ml flat or round bottom microwells, 2 ml flat bottom macrowells or in 25 cm^2 tissue culture flasks depending on the number of cells available.
6. If T cells have not been removed, then add a 1/100 volume of cyclosporine stock.
7. Incubate cultures at 37° C in a humidified atmosphere containing 5% CO_2.
8. Feed cultures twice weekly by removing half of the supernatant and replacing with fresh medium containing cyclosporine if this was used originally.
9. During the 1st and 2nd weeks of culture, care must be taken to maintain a cell concentration of at least 5 x 10^6 cells/ml when feeding with fresh medium. After cell lines have become established, the population doubling time ranges between 18 and 30 hrs with some variation in growth pattern and optimum cell concentration. In most cases, cultures should be maintained at no less than 5 x 10^5 cells/ml and no more than 4 x 10^6 cells/ml. Cyclosporine can be omitted from the medium after the cells have increased to 10-fold more than their original number.
10. Cultured cell lines may be lost for technical reasons. Therefore, it is suggested that all newly established cell lines be frozen as soon as possible. Cryopreservation of BLCL need not be carried out with a programmed freezer. Simpler procedures have been developed to freeze BLCL, which can be kept cryopreserved at -70° C for several months or in the vapor phase of liquid nitrogen for several years (see chapter on Cryopreservation).

Calculations

Not applicable.

Results

Growth of large refractile polygonal cells should become apparent within 2 weeks after starting the culture. Healthy cells grow in loose clumps.

Procedure Notes

Some investigators have reported difficulty establishing BLCL from black donors when cyclosporine is used to facilitate transformation (C. Johnson, personal communication). This difficulty can be circumvented by E-rosette depletion of T cells.

Limitations of Procedure

Cell lines are polyclonal at the beginning of culture and gradually tend to become monoclonal. Certain characteristics of cells can change spontaneously if cells are maintained continuously in culture for very long periods of time.

References

1. Bird AG, McLachlan SM, Britton S, Cyclosporine A promotes spontaneous outgrowth in vitro of Epstein-Barr Virus induced B-cell lines. Nature 289:300, 1981.
2. Conner BR, Pellegrino MA, Ferrone S, Glaser R, Lymphoid cell line identification and the detection of cross-contamination. In Vitro 16:446, 1980.

3. Martin PJ, Giblett ER, Hansen JA, Phenotyping human leukemic T-cell lines: enzyme markers, surface antigens, and cytogenetics. Immunogenetics 15:385, 1982.
4. Miller G, Lipman M, Release of infectious Epstein-Barr virus by transformed marmoset leukocytes. Proc Natl Acad Sci USA 70:190, 1973
5. Moore GE, Gerner RE, Kitamura J, Fjelde A, Lymphocyte cell lines derived from normal donors. In: *Proceedings of the Third Leukocyte Culture Conference*. WO Rieke, ed: Appleton-Century Crofts, New York; p 177, 1969.
6. Rickinson AB, Cellular immunological responses to the virus infection. In: *The Epstein-Barr Virus.* MA Epstein, BG Achong, eds.; John Wiley & Sons, New York; p 76, 1986.
7. Thorley-Lawson DA, Chess L, Strominger JL, Suppression of in vitro Epstein-Barr virus infection. A new role for adult human T lymphocytes. J Exp Med 146:495, 1977.
8. Tosato G, Blaese RM, Epstein-Barr virus infection and immunoregulation in man. Adv Immunol 37:99, 1985.

T Cell Cloning

Debra K. Newton-Nash and David D. Eckels

Purpose

Human T lymphocyte clones (TLCs) can be generated several ways. T cells can be grown in semi-solid matrices such as soft agar or methylcellulose and the resulting colonies, representing clones, can be plucked out and expanded in liquid cultures.[5,6] Clones can also be isolated using the single-cell deposition units found on fluorescence-activated cell sorters or by micromanipulation.[1] These methods will not be discussed and we will focus on limiting-dilution cloning, which is the method preferred by most laboratories.[2,4]

Cloning by limiting dilution is based on the distribution of small (i.e., limiting) numbers of cells at low density (i.e., high dilution) into many different wells of a tissue culture tray. Thus, there is a very small probability that any one well contains more than one cell. The actual probabilities can be calculated using Poisson statistics.[3] Plating cells at 0.3 cells per well yields a probability of 96.3% that any given well contains 0 or 1 cell. For arcane statistical reasons, if a clone is isolated from a well, the probability that it is derived from only a single progenitor cell is approximately 80%. In other words, 20 "TLCs" out of 100 will not be true clones. If it is important that a putative clone derives from a single cell, then subcloning is possible using the same approach. This might apply to the separation of two functions attributable to the same cell, cytotoxicity and proliferation, for example. Direct cloning by limiting dilution can be readily applied to cloning of specific T cells present within a primed population at relatively high frequency (i.e., alloreactive T cell cloning). The relatively low frequency with which antigen- or peptide-specific T cells are found within primed lines may necessitate plating initially at non-limiting dilution followed by subcloning of "cloids" at limiting dilution for the isolation of true T lymphocyte clones.

Several factors are problematic when cloning T cells. It is first desirable to provide a strong stimulus for T cell mitogenesis. The MLC or antigen-driven signals are more than adequate and will stimulate T-cells when provided as allogeneic stimulators or antigen plus presenting cells. In our hands, a growth hormone [T cell growth factor (TCGF) or interleukin-2 (IL-2)] is also required. Finally, as these cells are expanded with alternating exposure to antigens and TCGF, it is important to maintain sterility. Bacterial or fungal contaminations are obviously ugly. More insidious are problems with mycoplasma species, which can interfere with the growth and function of T cell lines. Therefore, careful technique is required as well as suitable antibiotics.

Specimen

Freshly drawn anticoagulated venous blood can serve as a source of PBLs. Alternatively, cryopreserved PBL or TLC can be used.

Reagents and Supplies

1. RPMI Complete Medium

Reagent	Volume	Final
RPMI 1640 medium	945 ml	n.a.
1M HEPES	25 ml	25mM
200mM L-glutamine	10 ml	2mM
40 mg/ml gentamicin sulfate	0.1 ml	4 µg/ml
10,000 U/ml heparin sodium	1.0 ml	10 IU/ml
100mM sodium pyruvate	10 ml	1mM
Penicillin-Streptomycin	10 ml	
10,000 U/ml penicillin		100 U/ml
10 mg/ml streptomycin		100 µg/ml

   a. Mix thoroughly and filter through 0.22 µ filter.
   b. Store in 500 ml aliquots at 4° C.
2. Pooled Human Plasma (PHP)
   See chapter on Preparation of T Cell Lines.
3. Fetal bovine serum (FBS)
   a. FBS is used for thawing and cryopreservation of cells. Screen in the MLC the same as PHP.
   b. Heat-inactivate at 56° C for 30 min.
   c. Filter through 0.22 µ. If it is difficult to filter, centrifuge at 400 x g for 30 min at room temperature and filter supernatant through 0.45 µ filter prior to filtration through 0.22 µ.
4. Ficoll-Hypaque (FH)

5. T cell growth factor (TCGF)
   See previous chapter (Preparation of T Cell Lines)
6. Thawing Medium (RPMI Complete Medium/10% FBS/1% Tylosin)
   a. Withdraw and discard 11 ml RPMI COMPLETE MEDIUM from a 100 ml flask.
   b. Add 10 ml of 0.22 µ filtered, heat-inactivated FBS (Biocell Labs) and 1.0 ml of Tylosin.
   c. Store at 4° C.
7. Culture Medium (RPMI/10% PHP/20% TCGF)
   a. Withdraw and discard 150 ml of RPMI Complete Medium from a 500 ml flask.
   b. Add 50 ml of 0.22 µ filtered PHP and 100 ml of 0.22 µ filtered TCGF.
   c. Store at 4° C.
8. Cryopreserved Cells
   a. Cryopreserve PBL and TLC using a controlled-rate freezing system. Freezing ramps are empirically determined for each sample type on a given system.
   b. Store frozen samples in the vapor phase of liquid nitrogen, not submersed, to minimize sample contamination.

Instrumentation/Special Equipment

1. Laminar flow hood
2. CO_2 incubator
3. Centrifuge
4. Controlled-rate freezing system
5. Liquid nitrogen freezer

Procedure

Thawing Cells

1. Let glass vials thaw at room temperature. Wipe the tops with 70% ethanol and let dry. Thaw Nunc vials in a 37° C waterbath.
2. Remove cells and dispense into an **empty** 15 ml polystyrene centrifuge tube.
3. Rinse vials with 0.5 ml of thawing medium 2-3 times, each time adding the rinse solutions to the cells.
4. Adjust the volume to 15 ml with thawing medium and invert the tube to insure dilution of residual DMSO present within the frozen sample.
5. Pellet cells by centrifugation at 200 x g for 10 min.
6. Pour off the supernatant and gently tap the bottom of the tube to resuspend cells.
7. Add 1-2 ml of desired medium (assay or culture medium), check for viability by trypan blue dye exclusion, count and adjust to appropriate cell concentration.

Priming Cells

1. Alloantigen priming. Combine responder cells with an equal volume of irradiated (3000 rads if PBL, 10,000 rads if B-lymphoblastoid cell lines) allogeneic stimulator cells at a final concentration of 5×10^5 cells/ml in culture medium. This can be done in 25 cm^2 tissue culture flasks or in 96-well, round-bottom trays. Incubate 5-7 days at 37° C in a humidified 5% CO_2/air environment.

2. Antigen-specific priming. Prepare PBLs at 5.0×10^5 cells/ml in culture medium. Prepare antigen at a two-fold higher concentration than optimum in culture medium. Combine 2.5 ml PBLs and 2.5 ml antigen in 17 x 100 mm polypropylene tissue culture tubes. Incubate 7 days at 37° C in fully humidified 5% CO_2/air.

Cloning Cells

1. Alloreactive T cell clone.
   a. Centrifuge primed cells over FH (200 x g for 10 min) to clear dead cells and debris. Remove cells from the interface, count and adjust to 30 cells/ml in culture medium.
   b. Prepare feeder cells consisting of irradiated stimulator cells at 1×10^6 cells/ml in culture medium.
   c. Combine primed cells and feeder cells 1:1, mix thoroughly and plate in 20 µl/well aliquots in sterile Terasaki trays. This ends up at approximately 0.3 primed T-cells/well and 1×10^4 feeder cells/well.
   d. Wipe small chambers with ethanol and line bottoms with paper towels moistened with autoclaved distilled water. Carefully stack Terasaki trays in chambers and cover loosely. Incubate 7-10 days at 37° C in fully humidified 5% CO_2/air.
   e. Score wells as positive or negative for proliferating cells under phase contrast microscopy.
   f. Transfer contents of positive wells to 200 µl cultures in 96-well, flat-bottom trays containing 1×10^5 cells/well of irradiated feeder cells in culture medium.
   g. Incubate 7 days at 37° C in humidified 5% CO_2/air.

2. Antigen-specific T cell clone.
   a. Centrifuge antigen-primed lines, count and adjust to 40 cells/ml in culture medium.
   b. Prepare autologous irradiated PBLs at 1 x 10^6 cells/ml in culture medium containing antigen at a two-fold higher than optimally stimulatory concentration.
   c. Follow steps c-g as for alloreactive T cell cloning and transfer, above, remembering to include antigen as stimulus in step f.
3. Antigen-specific T cell cloid.
   a. Prepare stimulators consisting of irradiated autologous PBLs at 1 x 10^6/ml in culture medium containing antigen at a two-fold higher concentration than optimum. Plate 100 µl stimulators + antigen into each well of a 96-well flat-bottomed tissue culture plate.
   b. Prepare primed cells at 1000 cells/ml in culture medium. Into wells containing stimulators + antigen, plate 100, 30, 10 or 3 µl to achieve final concentrations of primed cells of 100, 30, 10 or 3 cells/well. Add culture medium to bring the final volume in each well to 200 µl.
   c. Incubate plates at 37° C in fully humidified 5% CO_2 air for 7 days.
   d. Remove 100 µl medium from each well and replace with 100 µl fresh culture medium. Incubate 7 days at 37° C in humidified 5% CO_2/air.
   e. Score wells as positive or negative for proliferating cells under phase contrast microscopy. **Note:** T cell populations obtained in this manner should be referred to as cloids until clonality can be established by independent means. Subcloning by limiting dilution of T cell cloids may be required to derive cloned T lymphocytes.

Expansion of Clones

1. From 96-well trays, transfer contents of growing wells to 2 ml culture in 24-well trays containing appropriate stimulator cells in culture medium.
   a. Alloreactive T cell clones require 1 x 10^6 irradiated allogeneic PBL.
   b. Antigen-specific T cells clones require 1 x 10^6 irradiated autologous PBL suspended in culture medium containing antigen at optimum concentration.
2. Incubate 7 days at 37° C in humidified 5% CO_2/air. After the first 3 days, carefully remove approximately 1 ml from each well and replace with 1 ml fresh culture medium. Return to humidified 37° C, 5% CO_2/air incubator for the remainder of 7 day culture.
3. Maintain clones on a bi-weekly feeding schedule, providing stimulator cells (plus antigen when required) in culture medium weekly and fresh culture medium every 3 days thereafter.

Assay of TLCs

For functional assays of TLCs see chapters on Primed Lymphocyte Test or Cytotoxicity.

Results/Procedure Notes

1. No clones obtained in the limiting dilution stage or TLCs fail to expand.
   Reevaluation of priming conditions may be required to optimize antigenic stimulus or responder-stimulator combination required to sustain proliferation. Alternatively, check for subliminal infection.
2. TLCs not functional
   Ensure that appropriate positive controls were utilized and that assay conditions were adequate to assay for TLC function. Provided these conditions were satisfied, failure of a TLC to proliferate in the positive control may indicate the presence of mycoplasma infection. Expansion of the TLC in the presence of 1% Tylosin for 2-2½ weeks may rescue TLCs that are infected; however, we recommend that this treatment regimen not be used in long-term cultures to avoid selection of resistant forms of contaminants, which can be quite persistent, endangering all the cell lines in your laboratory and making you quite frustrated. Transient exposure to these agents, such as during thawing procedures, has worked well for us.

References

1. Bach FH, On getting a T cell clone and being assured you have one. Immunology Today 4:243, 1983.
2. Bach FH, Inouye H, Hank JA, Alter BJ, Human T lymphocyte clones reactive in primed lymphocyte typing and cytotoxicity. Nature 281:307, 1979.
3. Bailey NTJ, *Statistical Methods in Biology*. Hodder and Stoughton, London, 1959.
4. Eckels DD, Hartzman RJ, Characterization of human T-lymphocyte clones (TLCs) specific for HLA-region gene products. Immunogenetics 16:117, 1982.
5. Rozenszajn LA, Shoham D, Kalechman I, Clonal proliferation of PHA-stimulated human lymphocytes in soft agar culture. Immunology 29:1041, 1975.
6. Sredni B, Tse HY, Schwartz RH, Direct cloning and extended culture of antigen-specific MHC-restricted, proliferating T lymphocytes. Nature 283:581, 1980.

Propagation of Lymphoid Cells from Biopsies

Adriana Zeevi

Purpose

Although the outcome of organ transplants has markedly improved in recent years, allograft rejection is still a major problem. The histologic evaluation of biopsies from rejected organ transplants has shown infiltration of mononuclear cells. In human studies, characterization of these cells using immunohistochemical staining has shown a preponderance of T cells. Both CD4 (helper/inducer) and CD8 (cytotoxic/suppressor) subpopulations are present in the graft and express receptors for TAC as well as the HLA-DR antigen indicating that they are activated T cells. Although these studies provide information identifying different types of infiltrating T cells by cell surface markers, little is known as to either the functional characteristics or the specificity of allorecognition.

Initially, numerous investigators attempted to study intragraft events by monitoring lymphocyte populations in the peripheral blood.[1-3] However, other investigators showed that monitoring of the peripheral blood population has limited value and does not reflect the events occurring in the graft.[4,5]

A more direct approach to study the functional characteristics of infiltrating T cells is to isolate T cells from the graft. This can be accomplished by enzymatic digestion of rejected allografts[6] or by mechanical extraction of the cells infiltrating these allografts.[7,8] The first method may introduce artifacts due to the enzymatic digestion while the latter is limited by the small number of cells obtained from the biopsy material. An attractive approach to learn more about the types of cells involved in the allograft response is the propagation of lymphocytes from transplant biopsies.

The technique is based on the concept that transplant biopsies undergoing an allograft response would be infiltrated by activated T cells capable of responding to interleukin 2 (IL-2) *in vitro*. These principles have been applied to study infiltrating T cells in renal,[9-11] cardiac[12,13] and hepatic[14] allografts. Recombinant IL-2 (rIL-2) is generally used to propagate lymphocytes from allograft tissues and the concentration of rIL-2 has ranged from 5-300 U/ml.

The rIL-2 concentration is important since low doses may not be sufficient for all T cell subsets ($CD4^+$ vs. $CD8^+$) whereas high doses may induce proliferation of other non-T cell types such as lymphokine activated killer cells.[15]

Mayer et. al.[9] and Miceli et. al.[10] reported growth of lymphoid cells from renal core biopsies cultured in tissue culture medium supplemented with rIL-2. However, once cellular outgrowth was noted, they expanded their cell lines with either pooled allogeneic feeder cells or Epstein Barr Virus (EBV)-transformed donor lymphoblastoid cells, respectively. The potential drawback of this methodology is that by expanding the initially established T cell line with alloantigen prior to functional testing, the reactivity pattern of these cells may be altered. In our method we have avoided this potential problem by maintaining biopsy derived cultures for 14-16 days in rIL-2 only prior to testing for donor-specific alloreactivity.

The size of the biopsy tissue used for culture may influence the frequency of lymphocyte growth and at least four 1 mm^3 fragments are needed from an endomyocardial biopsy (EMB) to propagate graft infiltrating lymphocytes.[16]

Endomyocardial Biopsy Culture Protocol

Purpose

Our lab has demonstrated that lymphocyte growth from histologically negative endomyocardial biopsies is associated with an earlier appearance and higher incidence of subsequent histological rejection of the cardiac allograft.[17] Furthermore, the alloreactivity of biopsy-grown cells toward donor cells presents an additional risk-factor for rejection. These findings indicate that a considerable proportion of heart transplant biopsies are already infiltrated by alloactivated lymphocytes mediating allograft rejection at times when biopsy histology is considered negative for rejection. Thus, the *in vitro* propagation of lymphocytes from histologically negative biopsies has potential usefulness as a laboratory assay to predict cardiac transplant rejection.

Specimen

1. An endomyocardial biopsy is placed in a jar containing sterile physiological saline.
2. Approximately 10-20 ml of patient blood in heparin accompanies each biopsy specimen. The whole blood for adults is obtained in 1-2 green topped (containing heparin) 10 ml vacutainer tubes. Pediatric samples are obtained in 1-2 green topped, 5 ml vacutainer tubes.

Instrumentation

1. Laminar flow hood
2. Centrifuge

3. Eppendorf adjustable digital pipettor (10-100 µl)
4. Microscope
5. CO_2 and 37° C water jacket incubator
6. ^{137}Cesium gammacell irradiator
7. Drummond portable Pipet-Aid
8. Hamilton repeating dispenser
9. Hamilton gas-tight syringe, 2.5 ml capacity
10. Hamilton gas-tight syringe, 0.5 ml capacity

Reagents

1. RPMI 1640 medium with L-glutamine
2. N-2-Hydroxyethylpiperazine-N'-2-Ethanesulfonic acid (HEPES) buffer solution 1M
3. Gentamicin reagent solution 10 mg/ml
4. Eosin B
5. Human AB serum
6. Recombinant interleukin-2 (rIL-2)
7. Ficoll-Paque – store between 4° C and 25° C
8. Thymidine, methyl-^{3}H (^{3}H-Tdr) (5 mCi in 5 ml of sterile, aqueous solution, approximately 33 Ci/mM)–ICM

Procedure

Preparation of Tissue Culture Medium

1. Prepare Tissue Culture Medium (TCM) supplemented with 5% human AB serum. Add to 500 ml bottle of RPMI + 12.5 ml of HEPES Buffer Solution, 2.8 ml of Gentamicin Reagent Solution (10 mg/ml). Mix and store at 4° C. Prepare solution of 5% human serum in TCM, filter through 0.2 µ filter and store at 4° C.
2. Prepare IL-2 (20 IU/ml) in TCM supplemented with 5% human serum.

Peripheral Blood Lymphocytes

1. Empty contents of 10 ml green topped tube into sterile 50 ml centrifuge tube.
2. Add sterile RPMI to blood until total volume equals 40 ml. Mix well.
3. Add 12.5 ml of Ficoll-Paque and underlay by air pressure.
4. Spin for 30 min at 350 x g (approximately 1200 rpm).
5. Remove interface without touching red blood cell layer.
6. Add sterile RPMI to the interface and mix well. Spin for 15 min at 350 x g (approximately 1200 rpm).
7. Discard supernatant. Add 2 ml of TCM supplemented with 5% serum.
8. Count number of cells per ml on a hemacytometer and adjust concentration to 2 x 10^5 cells/ml.
9. Irradiate the isolated peripheral blood lymphocytes (PBLs) at 4000 rads. These are the autologous PBLs to be used as feeder cells in the heart biopsy culture.

Endomyocardial Biopsy Culture

1. Place the biopsy pieces in the small (35 x 10 mm) sterile petri dish using a sterile transfer pipette.
2. Add enough transport medium (or sterile physiological saline) to swirl biopsies around.
3. Use sterile 20 G x ½ inch needles to tease the biopsies apart in the petri dish. Try to use all of the biopsy pieces. Add one (1) biopsy piece per well in a 96-well U-bottomed plate.
4. Add 100 ml of 20 U rIL-2 medium to each well containing a biopsy piece.
5. Add 50 ml of autologous irradiated feeder PBLs (concentration of 2 x 10^5/ml) to each well containing a piece of biopsy, and also to 3 wells with no pieces of biopsy to be used as a control. Add 100 ml of 20 U rIL-2/ml medium to each of these control wells.
6. Place covered 96-well plate into 37° C + 5% CO_2 incubator.
7. Note each biopsy in the heart biopsy log.
8. Monitor the biopsy cultures every Monday, Wednesday, and Friday under the inverted microscope for lymphocyte growth and note in the heart biopsy log.
9. Feed the biopsy cultures every Monday, Wednesday, and Friday with fresh TCM supplemented with rIL-2. Note positive cultures, splitting as necessary to maximize cell growth.
10. Continue to expand cultures in successively larger well culture plates (48 well, 24 well) until sufficient numbers of cells are generated to perform functional studies.
11. Evaluate lymphocyte cultures for donor-specific alloreactivity in the three day PLT assays. The testing should use cultures expanded for less than 16 days since cultures expanded for longer periods frequently lack PLT reactivity.
12. Further propagate lymphocyte cultures exhibiting donor-specific alloreactivity in the presence of donor irradiated feeder cells.

Hepatic Biopsies

1. Incubate liver biopsies in the presence of rIL-2 only, since this system apparently does not require feeder cells for initial lymphocyte propagation.
2. Replenish biopsies having increased bile concentration daily for the first 3-4 days with fresh TCM supplemented with rIL-2. Use of rIL-2 rather than supernatants of lectin-activated cultures avoids lectin activation.
3. Discard all biopsies lacking lymphocyte growth after two weeks.

Troubleshooting

Lymphocyte cultures propagated from transplant biopsies are tested for donor-specific alloreactivity in Primed Lymphocyte Test (PLT) assays. Prior to testing, the lymphocyte cultures do not receive IL-2 for 3 days. This is necessary to reduce background proliferation of these cultured cells. Whenever feeder cells are used to maintain these lymphocyte cultures, the functional assays should never be performed prior to 7 days after the last addition of feeder cells.

1. Human AB serum can be toxic to the cells and prevent their growth. The AB serum is quality control tested every time there is a company or lot number change.
2. The concentration and quality of rIL-2 may vary. The rIL-2 is quality control tested every time there is a company or lot number change.
3. Contamination may occur if biopsies are not performed and handled under sterile conditions. Cultures must carefully be observed for contamination.

Quality Control (QC) of Human Recombinant Interleukin-2

Preparation of Working Stock Solution

1. Reconstitute 1 bottle (1 x 10^6 units/bottle) with 1 ml of sterile, distilled water (1 x 10^6 units per ml).
2. Dilute with 9 ml of 1X PBS + 1% BSA (working solution: 6.8 g of NaCl; 1.48 g of Na_2HPO_4; 0.43 g of KH_2PO_4, and 10 mg of BSA in 1000 ml of distilled water, pH adjusted to between 7.2 and 7.4) to obtain 10 ml of rIL-2 working stock solution at 1 x 10^5 units/ml.
3. Aliquot 1 ml samples into 10 freezer vials. Label vials with contents (rIL-2), concentration (1 x 10^5 units/ml), and date. Store working vial at 4° C and all other vials at -70° C until needed.
4. Add desired amount of rIL-2 working stock solution to 100 ml of 5% AB-supplemented TCM and filter.
5. A quality control (QC) assay must be performed on each new shipment of human rIL-2. This serves a two-fold purpose: (1) to test the potency of the rIL-2 as shipped from the company, and (2) to determine the desired amount of rIL-2 working stock solution to add to TCM in order to ensure optimum lymphocyte proliferation.

Setting Up the Assay

1. Obtain a cell line that has been grown in a previously acceptable concentration of human rIL-2 (such as normal PBL) to use as the responder in this assay.
2. If the cells have been frozen, thaw the vial(s) in warm water and resuspend in 8-10 ml of 5% AB-supplemented TCM.
3. Spin for 10 min at 350 x g (approximately 1200 rpm).
4. Decant supernatant and resuspend in 1 cc of 5% AB-supplemented TCM to count cells.
5. Adjust concentration of cells to 2 x 10^5 cells/ml.
6. Make several concentrations of rIL-2 (5 units/ml, 10 units/ml, 20 units/ml, etc.) to test lymphocyte proliferation.
7. Plate a row of 5% AB-supplemented TCM (100 ml per well) in a 96-well plate to serve as a negative control for the rIL-2 concentrations.
8. Plate a second row (directly beneath the first row) of the cell line at the adjusted concentration of cells per ml (100 µl/well = 2 x 10^4 cells per well).
9. Plate several columns (duplicate or triplicate), the first column containing 5% AB-supplemented TCM serving as a negative control for the cell line. The other columns will contain the various concentrations of rIL-2.
10. Incubate the 96-well plate for 3 days (72 hrs) total. At 48 hrs, pulse the plate with 1 mCi of 3H-Tdr per well (dilution of 1 µCi/10 ml).
11. Continue incubation for another 18-24 hrs. Uptake and incorporation of ^{3}H-Tdr will occur in proliferating lymphocytes and can be measured on a beta counter in cpms.
12. Analyze data carefully. The optimum concentration of rIL-2 added to TCM should be the lowest concentration that achieves the highest cpms (minus background counts from negative controls).

References

1. Es A, Meyer C, Oljans P, Tanke H, Esl V: Mononuclear cells in renal allografts, correlations with peripheral blood T lymphocyte subpopulations and graft prognosis. Transplantation 37:134, 1984.
2. Ellis T, Berry C, Mendez-Picon G, Goldman M, Lower R, Lee H, Mohanakumar T: Immunological monitoring of renal allograft recipients using monoclonal antibodies to human T lymphocyte subpopulations. Transplantation 33:317, 1982.

3. Devineni R, McKenzie N, Keown P, Stiller C, Hellstrom A, Banerjee D: Immunologic monitoring in cardiac transplantation. Transplant Proc 16:1576, 1984.
4. Thompson J, Carter N, Bolton E, McWhinnie D, Wood R, Moris P: The composition of the lymphocytic infiltrate in rejecting human renal allografts is not reflected by lymphocyte subpopulations in the peripheral blood. Transplant Proc 17:556, 1985.
5. Shionozaki F, Kondo T, Fujimaura S, Nakada T: Technical establishment and detection of rejection in rat lung transplantation. Transplant Proc 17:244, 1985.
6. Tilney N, Kupiec-Weglinski J, Heidecke C, Lear P, Sromm T: Mechanisms of rejection and prolongation of vascularized organ allografts. Immunol Rev 77:185, 1984.
7. Moreau J, Peyrat M, Vie H, Bonneville M, Soulillou JP: T Cell colony-forming frequency of mononucleated cells extracted from rejected human kidney transplants. Transplantation 39:646, 1985.
8. Nikaein A, McQueen K, Boyer B, Landesberg R, Ryan DH, Insel RA: Functional characterization of renal infiltrating cells following allograft nephrectomy. Transplant Proc 19:398, 1987.
9. Mayer T, Fuller A, Fuller T, Lazarovits A, Boyle L, Kurnick J: Characterization of in vivo-activated allospecific T lymphocytes propagated from human renal allograft biopsies undergoing rejection. J Immunol 134:258, 1985.
10. Miceli C, Metzgar R, Chedid M, Ward F, Fin O: Long-term culture and characterization of alloreactive T cell infiltrates from renal needle biopsies. Human Immunol 14:295, 1985.
11. McKenna RM, Heiman D, Rush DN, Jeffery JR: Limiting dilution analysis of the frequency of functional T cells in human renal allograft fine needle aspirates. Transplant Proc 20:207, 1988.
12. Zeevi A, Fung J, Zerbe T, Kaufman C, Rabin B, Griffith B, Hardesty R, Duquensoy R: Allospecificity of activated T cells grown from endomyocardial biopsies from heart transplant patients. Transplantation 41:620, 1986.
13. Pfeffer PF, Foerster A, Tveter AK, Simonsen S, Froysaker T, Thorsby E: Donor-specific cytotoxic T cells recovered from transvenous biopsies after clinical heart transplantation. Transplant Proc 20:306, 1988.
14. Fung J, Zeevi A, Starzl T, Demetris A, Iwatsuki S, Duquesnoy R: Functional characterization of infiltrating T lymphocytes in human hepatic allografts. Human Immunol 16:182, 1986.
15. Grimm EA, Robb RJ, Roth JA, Neckers LM, Lachman LB, Wilson DJ, Rosenber SA: Lymphokine-activated killer cell phenomenon. J Exp Med 158:1356, 1983.
16. Saidman SL, Demetris AJ, Zeevi A, Duquesnoy RJ: Propagation of lymphocyte infiltrating human liver allografts: Correlation with histologic diagnosis of rejection. Transplantation 49:107, 1990.
17. Weber T, Kaufman C, Zeevi A, Zerbe T, Hardesty R, Kormos R, Griffith B and Duquesnoy RJ: Lymphocyte growth from cardiac allograft biopsy specimens with no or minimal cellular infiltrates: Association with subsequent rejection episodes. J Heart Transplantation 8:233, 1989.
18. Weber T, Kaufman C, Zeevi A, Zerbe T, Hardesty R, Kormos R, Griffith B, Duquesnoy R: Propagation of lymphocytes from human heart transplantation biopsies: Methodologic considerations. Transplant Proc 20:176, 1988.

The Mixed Lymphocyte Culture (MLC) Test

Eric M. Mickelson, Leigh Ann Guthrie, and John A. Hansen

Purpose

The mixed lymphocyte culture (MLC) reaction is an in vitro test of lymphocyte recognition and proliferation.[1,3] The assay represents a functional measure of cellular immunity in which T lymphocytes from one individual are induced to proliferate when stimulated by mononuclear leukocytes from a different, or allogeneic, individual. The primary activation signals for the MLC reaction are provided largely by polymorphic determinants on class II (HLA-D region) molecules, although certain subsets of T cells are able to respond to class I determinants. In quantitative terms, most of the proliferating cells that are directly measured in a primary MLC are those responding to class II determinants. By clonal analysis, however, it can be clearly shown that some T cells (usually $CD8^+$) are able to proliferate in response to class I determinants. The recognition of (foreign) class II alloantigen and the ensuing T cell activation that occurs in MLC are thought to represent an in vitro model of the afferent phase of an in vivo allograft reaction.[8,29]

Because the MLC assay involves the logarithmic expansion of multiple clones of alloactivated T cells measured by incorporation of radio-labeled nucleotide, careful attention to preparation of the cultures at the time of plating (day 0) is critical to assuring a meaningful readout at the time of maximum cellular proliferation (day 6). If a quantitative evaluation of lymphocyte proliferation is to be achieved (i.e., distinguishing strong reactions from weak reactions), the conditions for the assay must be established in a manner that will provide for linear response rates over a predetermined period of time. The selection of labeling time and duration are important considerations in quantifying the proliferative response. In order to define what constitutes a strong vs. a weak response, each laboratory must establish its own data base reflecting the performance of the MLC assay under the standard conditions used in that laboratory. If such a data base is derived from the testing of normal family members whose HLA-D region disparity or similarity are clearly known, a very useful reference standard is generated, in which the relative strength of an MLC response, and hence the degree of HLA-D region compatibility, between two cells of unknown HLA-D phenotype can be more accurately assessed.

The MLC serves as a cellular "crossmatch" and may provide information about cellular recognition events that may not be discernible by serologic or DNA typing methods.

Specimen

Freshly drawn anticoagulated venous blood is required for MLC testing. Volumes required may vary depending on the age of the individual, absolute lymphocyte count and expected size of the test; 20 ml is normally sufficient. Containers must be clean, sterile, and clearly labeled with the subject's name, relationship to patient and date of draw. Specimens should be kept at room temperature and should arrive in the laboratory within 24 hrs of being drawn.

Specimens are unacceptable for testing if they are more than 48 hrs old, clotted, or have been stored on ice. Specimens should be rejected if they arrive in broken or leaking containers, are labeled improperly or are in syringes with needles attached.

Reagents and Supplies

1. Lymphocyte separation medium (LSM)
2. Complete medium
   a. 100 ml RPMI 1640 medium with 25mM HEPES buffer
   b. 100 µg/ml penicillin
   c. 100 µg/ml streptomycin (a commercially prepared antibiotic solution may be substituted)
   d. 10 IU/ml heparin (optional)
   e. Pooled human serum (PHS) to make a 10-20% solution
   f. Hanks Balanced Salt Solution (HBSS)
3. Pooled human serum
   a. Serum from 10-20 normal healthy untransfused male donors
   b. Heat-inactivate the serum at 56° C for 30 min.
   c. Test each serum sample in MLC for ability to support growth and screen each against an appropriate cell panel in an appropriate assay to ensure that the serum donor does not have anti-lymphocyte antibodies.
   d. Pool the serum and dispense in appropriate sized tubes.
   e. Freeze and store at -20° C (acceptable) or -80° C (optimal)
   f. Test each new serum pool in MLC for optimal concentration as medium supplement. Suggested concentrations are 10, 15 and 20%.

4. Radiation Source
   An irradiation machine, usually containing a gamma emitting radiation source, is a convenient method of inactivating stimulator cells. The most common source is ^{137}Ce; some machines may utilize a ^{60}Co or other source.
5. Mitomycin-C (if radiation source is not available)
   a. Mitomycin-C
   b. Sterile distilled water
      1) Dilute stock mitomycin-C to 0.25 mg/ml
      2) Store at 4° C shielded from light
6. ^{3}H-Thymidine (^{3}H-Tdr)
   a. ^{3}H-Tdr from manufacturer
   b. RPMI 1640 medium
      1) Dilute 1 ml of 1 mCi/ml stock ^{3}H-Tdr solution with 24 ml of culture medium, yielding a working solution of 40 µCi/ml. 25 µl of diluted ^{3}H-Tdr will therefore contain 1 µCi. Specific activities of 2.0, 5.0 or 6.7 Ci/mM are routinely used.
      2) Store at 4° C.

Instrumentation

1. Laminar flow hood
2. Centrifuge
3. Repeating dispensers for delivering volumes from 25-100 µl
4. Microscope or Coulter Counter
5. CO_2 incubator
6. Radiation source (optional)
7. Multiple sample harvester
8. Liquid Scintillation Counter
9. Data reduction and processing system (optional)
10. Refrigerator
11. Freezer (-20° C)
12. Liquid nitrogen freezer

Calibration

Instruments such as repeating dispensers and Coulter Counters used in the MLC assay must be calibrated periodically, either by laboratory personnel or by qualified professional technicians, in order to assure that delivered volumes and cell counts are consistent. Liquid Scintillation Counters are equipped with standards that should be included each time the machine is operated and the resultant counts per minute should be recorded.

Quality Control

Due to the length of the culture period and variability of culture conditions present in the MLC assay, it is particularly dependent on rigorous quality control measures for reagents and equipment including:

1. Each individual lot of serum should be tested for growth support capability before pooling and freezing; one bad sample can ruin an entire batch of pooled serum.
2. Incubator temperatures must be monitored carefully, with at least one mercury thermometer kept inside the chamber as a double check on the temperature recording device built into the incubator. Even slight deviations from 37° C can drastically alter cell growth characteristics.
3. The harvest machine should be first thoroughly flushed with water before each set of plates is to be harvested. Then, two or more sets of filter disks (water only) can be harvested as background controls. These background controls should be counted in the scintillation counter along with the actual MLC assays for that day to provide a control for the harvest machine efficiency and cleanness. Another set of background controls (water only) can be run after the MLC harvesting is complete for that day.
4. All new batches of reagents should be run in parallel with existing lots and the new lot numbers should be recorded as they are used.

Procedure

1. Bring all liquid reagents to room temperature before use. All procedures through step 10 (incubation) are carried out at room temperature.
2. Aseptically, draw heparinized blood (20 units heparin/ml blood) from each person to be tested and mix thoroughly in the syringe (see chapter on Specimen Acquisition).

3. Dilute the whole blood 1:2 with HBSS. Separate the peripheral blood mononuclear cells (PBMC) by centrifugation over LSM according to the procedure described in chapter on Density Gradient Isolation of PBL. If small volumes of blood are being processed, the diluted blood may be layered above the LSM; if larger volumes (>10 ml) are being processed, the LSM may be underlayered beneath the blood.
4. Remove the PBMC from the LSM interface, dilute 1:2 with HBSS and centrifuge at 500 x g for 10 min.
5. Decant the supernatant, resuspend the cells in 4-5 ml HBSS and centrifuge for 5 min at 180 x g. Decant and repeat wash.
6. After the second wash, resuspend the cells in 3 ml of complete medium.
7. Perform white cell count and check for percentage of mononuclear leukocytes. Determine viability using Trypan blue or other vital stain.
8. Using complete medium, dilute the cell suspensions to a final concentration of 5 x 10^5 mononuclear leukocytes/ml. Prepare two suspensions for each individual tested: one to be used as stimulator cells and one as responder cells.
9. Inactivate the stimulator cells by:
   a. Exposure to 1500-3000 R from an irradiation source, or
   b. Incubation with mitomycin-C:
      1) Add 0.025 mg mitomycin-C (0.5 mg/ml) to each 1 ml of cell suspension, incubate 20 min in a 37° C water bath.
      2) Wash twice in HBSS and resuspend in complete medium.
      3) Adjust cell count to 5 x 10^5 up to 1 x 10^6 cells/ml with complete medium.
10. Distribute stimulating and responding cells in triplicate to the wells of round bottom microtiter plates using a repeating microliter pipette or syringe. Each well should receive 100 µl of stimulating cells and 100 µl of responding cells (5 x 10^4 up to 1 x 10^5 cells each). Three types of cultures should be set up:
    a. Allogeneic cultures, containing responding cells from one individual and stimulating cells from another.
    b. Autologous control cultures, containing stimulating and responding cells from the same individual.
    c. Double irradiation control cultures, containing stimulating cells from two different individuals, to assess the efficacy of inactivation.
    d. In addition to the above combinations, cultures containing responding cells in medium alone and cultures containing responding cells with phytohemagglutinin (PHA) may also be set up. These are not essential, but may give additional information about the behavior and response characteristics of the cell populations being tested. An example of an MLC test, including patient, family members and unrelated controls, is shown in Figure 1.

Responders	**Stimulators**			
	$Patient_x$	$Sibling_x$	$Sibling_x$	$Father_x$
Row A Patient	O O O	O O O	O O O	O O O
	$Mother_x$	$Control_x$	$Control_x$	$Pool_x$
Row B Patient	O O O	O O O	O O O	O O O

Row C.....

RowD.....

Figure 1. Format for setting up a family MLC

11. Cover the culture plates with the plastic lid and place in the incubator at 37° C in a humidified atmosphere of 5% CO_2/air. Incubate for a total of 138 hrs (approximately 6 days). The peak of proliferation occurs on day 6 to 7 (see Figure 2). Check to make sure that the humidity is sufficient to prevent evaporation of culture fluid from the wells.
12. After 120 hrs (5 days), remove culture plates from the incubator and add 1 µCi (0.025 ml) of tritiated thymidine to each well. Return plates to incubator.
13. After the 138 hr culture period (18 hrs following addition of the radiolabel) remove the culture plates from the incubator. Harvest immediately, or seal the plates with pressure sensitive film and place in the refrigerator at 4° C, where they may be kept for up to one week.
14. A variety of automated harvest machines are commercially available with which to harvest mixed cultures at the end of the incubation period and prepare them for scintillation counting. Consult the instruction manual of the specific machine being used for appropriate procedure to be followed in harvesting.
15. After the cultures have been harvested and the DNA residue captured on filter disks, transfer the disks to an appropriate counting vial, add scintillation fluid (as little as 1 ml of scintillation fluid per vial may be sufficient) and count in a scintillation counter. The appropriate length of counting time for each sample can be determined by consulting the scintillation counter procedure manual. This step may vary depending upon the scintillation counter being used, e.g., LKB beta counter requires bags instead of vials.

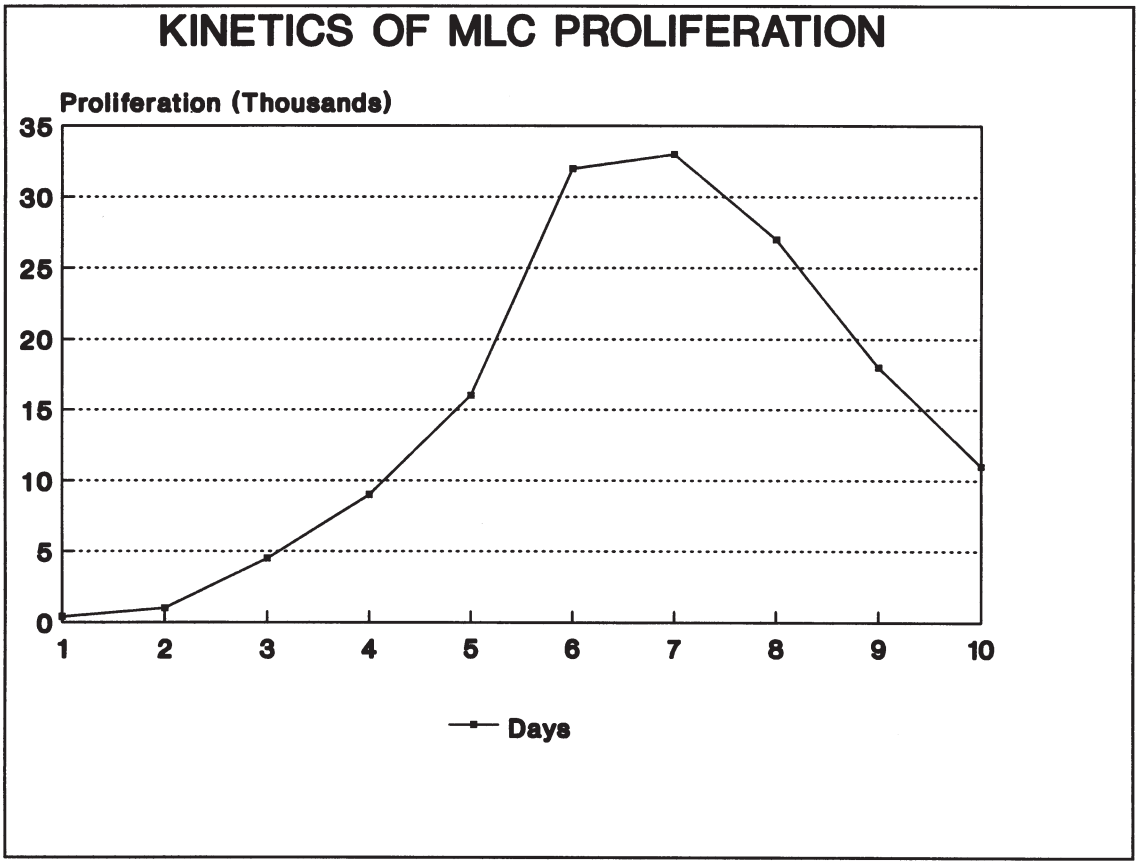


Figure 2. Time course kinetics of a primary MLC response. Incorporation of tritiated thymidine by proliferating cells usually reaches a maximum at day 6-7.

Calculations

The results from a typical MLC test as determined by scintillation spectrophotometry are expressed in raw form as counts per minute (cpm) of disintegration of the tritium radioisotope. In order to interpret these results in an objective manner, the cpm must be transformed, or reduced, to yield data that can be more easily quantified and analyzed.[27] The two most common methods of achieving data reduction are:

1. Stimulation index (SI): a simple ratio between the cpm obtained in one allogeneic combination, divided by the cpm obtained in the appropriate autologous control; also called an "index of transformation."

$$SI = \frac{\text{experimental MLC}}{\text{autologous MLC}}$$

2. Relative Response (RR): the ratio between the net cpm in an allogeneic combination (A + Bx) and the net cpm in a maximally stimulated combination (A + Unrelated x)

$$RR = \frac{\text{allogeneic MLC} - \text{autologous MLC}}{\text{maximum MLC} - \text{autologous MLC}}$$

The reference response value is equated to the maximum response obtained for the particular responder cell in the experiment; this is usually provided by one of the individual unrelated control cells or by the pool of unrelated control cells. The ratio is usually multiplied by 100 to yield a "percent RR value."

Results

An example of a typical family MLC experiment, with raw cpm data and calculated SI and RR values, is shown in Table 1.

Table 1. Family MLC Test. "U1" and "U2" indicate two individual unrelated control cells; "pool" indicates a pool of four different unrelated cells selected to be maximally disparate for HLA-D. The HLA haplotypes of each family member are designated *a*, *b*, *c* and *d*. Data for each combination are given as mean CPM (top), SI (middle) and RR (lower number).

	STIMULATOR CELL								
RESPONDER CELL	**Patient X**	**Sib 1X**	**Sib 2X**	**Sib 3X**	**Mother X**	**Father X**	**U1 X**	**U2 X**	**Pool X**
Patient a/c	(150) 1.0 0	160 1.1 0	32,518 216.8 46	61,297 408.7 86	40,271 268.5 57	55,419 369.5 78	60,882 405.9 86	71,004 473.4 100	63,409 422.7 89
Sibling 1 a/c	602 1.0 0	(593) 1.0 0	48,217 81.3 54	80,492 135.7 91	50,883 85.8 57	44,017 74.2 50	79,327 133.8 90	88,100 148.6 100	62,499 105.4 71
Sibling 2 b/c	37,800 31.5 44	47,650 39.7 55	(1,201) 1.0 0	48,290 40.2 56	27,300 22.7 31	31,692 26.4 36	80,226 66.8 94	68,490 57.0 80	85,117 70.9 100
Sibling 3 b/d	46,332 58.8 58	86,636 109.9 109	40,737 51.7 51	(788) 1.0 0	39,117 49.6 49	45,507 57.8 57	79,224 100.5 100	72,239 91.7 91	78,844 100.1 100
Mother a/b	21,103 44.3 29	40,010 84.1 57	39,954 83.9 56	38,807 81.5 55	(476) 1.0 0	57,311 120.4 81	40,809 85.7 58	62,711 131.7 89	70,442 148.0 100
Father c/d	33,393 12.3 43	37,717 13.4 49	50,771 18.7 68	41,555 15.3 55	77,398 28.5 105	(2,713) 1.0 0	60,004 22.1 81	73,877 27.2 100	60,321 22.2 81
U1	47,100 146.7 52	91,129 283.9 101	67,816 211.3 75	91,006 283.5 100	69,641 217.0 77	83,569 260.3 92	(321) 1.0 0	65,010 202.5 72	90,557 282.1 100
U2	31,896 35.8 24	87,403 98.2 94	41,307 46.4 44	89,713 100.8 96	59,546 66.9 63	70,883 79.6 76	93,334 104.9 100	(890) 1.0 0	88,410 99.3 95

In the example given in Table 1, there is an HLA identical or zero haplotype (0h) incompatible sibling combination (patient + sibling 1), several one haplotype (1h) incompatible combinations (e.g., patient or sibling 1 + sibling 2; any parent-child combination) and a two haplotype (2h) incompatible combination (patient or sibling 1 + sibling 3). Note that the RR values obtained from these combinations are close to 0% (0h), to 50% (1h) or to 100% (2h), depending upon the degree of HLA-D region incompatibility between the reacting cell populations. This indicates that, for 0h incompatible sibling combinations, T cell activation does not occur in MLC, while for 1h and 2h incompatible combinations approximately 50% or 100% of responding T cells, respectively, are activated to proliferate. This same relationship between incompatibility for 0, 1 or 2 haplotypes and MLC reactivity can be demonstrated using the SI calculation. Thus, by expressing MLC data in terms of an RR or an SI value, an approximation of the degree of HLA-D compatibility or incompatibility between the reacting cell populations can be achieved.

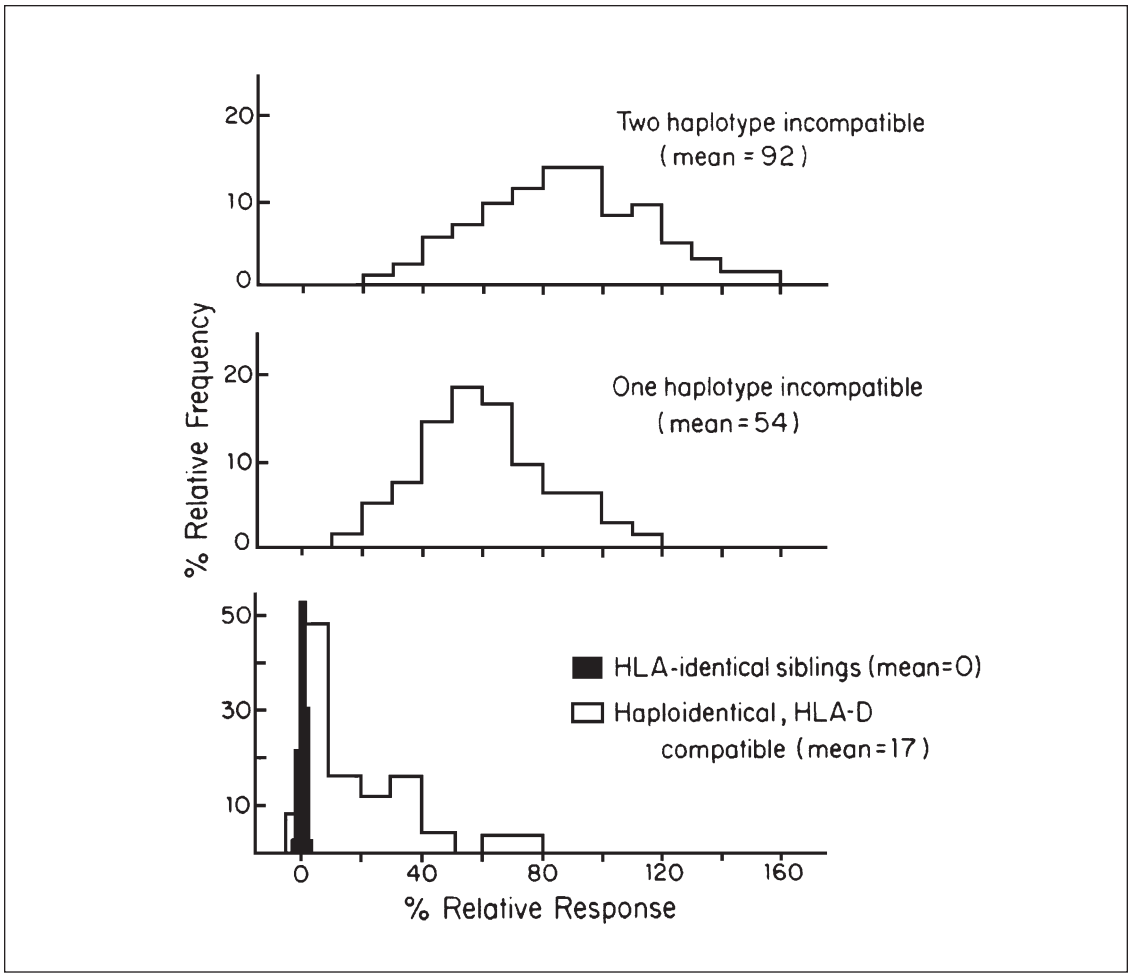


Figure 3: Frequency histrograms showing %RR values derived from testing family member pairs known to differ by 0, 1 or 2 HLA haplotypes. A fourth type of combination, those 1 haplotype incompatible pairs who are known to be HLA-D compatible for their nonshared haplotypes, is shown in the lower figure.

Figure 3 shows representative SI and RR data derived from testing more than 500 pairs of 0h, 1h and 2h incompatible family members in MLC. In these experiments, the %RR values were derived by using as reference response the mean stimulation provided by two individual unrelated control cells and a pool of four different unrelated individuals. The mean %RR derived from testing 2h incompatible combinations is 92%, for 1h incompatible combinations 54%, and for 0h incompatible combinations (HLA identical siblings), 0%. The lower portion of the figure also displays the results of testing 1h incompatible combinations that are HLA-Dw compatible (by testing with HTC) for their unshared HLA haplotype (mean RR = 17%). This type of data, accumulated within each laboratory performing MLC testing, provides a standard that can be used to interpret clinical MLC assays in which the degree of HLA-D incompatibility between the reacting cell populations is unknown.

If desired, data from an MLC test can be further reduced by a "stimulatorwise" or "vertical" normalization; e.g., normalizing the data a second time to account for the varying ability of the stimulator cells to stimulate. This second normalization step produces a double-normalized value, or DNV. For a more extended discussion of the DNV procedure, see the chapter on HLA-Dw typing as well as references 20, 24 & 28.

Procedure Notes

1. **Troubleshooting**
   Problems that arise in the MLC assay can usually be traced to technical conditions of the assay itself or to the quality and condition of the leukocytes that are being cultured. These problems usually lead to poor growth characteristics of the cultured cells. The former type of problem can often be avoided by careful quality control measures in the laboratory as outlined in a preceding section. The latter type of problem, often a result of culturing cells obtained from patients with leukemia or renal failure, can be more difficult to control and represents a continual source of variability in the MLC assay. This problem may be overcome by isolating resting lymphocytes by

a variety of methods such as Dynabeads, Lympho-Kwik, monoclonal antibodies, etc. (see the related chapters for cell isolation).

2. **Technical Considerations**
   a. Serum
      The most common technical problem that occurs in MLC assays is poor quality of the serum used to supplement the culture medium, usually manifesting itself as suboptimal cell growth characteristics; i.e., low cpm. If the individual lots have been carefully tested for growth support capability, the most likely source of poor quality serum is improper storage. In general, serum should not be stored longer than three months at -20° C; it may be stored for longer periods at -80° C, but should be continually checked for quality. Make sure that the quantity of serum that is used for routine MLC assays is optimal: 20% volume (v/v) serum/medium is not necessarily twice as good, or even better than, 10% v/v serum/medium. Serum that has been derived from recalcified plasma will often tend to produce a calcium chloride precipitate after 1-2 months of storage. This precipitate does not appear to be toxic to the growing lymphocytes in culture, but may form a deposit on the plate that may interfere with cell to cell interaction or cell harvesting procedures.
   b. Temperature
      Poor cell yield following ficoll-hypaque separation or poor growth (low cpm) of mixed cultures may result from suboptimal temperature conditions. All procedures in blood cell separation, processing and culture set-up are carried out at room temperature. Care should be taken during the LSM separation phase to insure that the diluted blood is at room temperature. Blood specimens that have been shipped into the laboratory may arrive cold, and should be brought to 22° C before processing. If LSM is stored in the refrigerator, make sure that it is brought to 22° C (not to 37° C in a water bath) before use. Incubator temperatures should be monitored carefully, as previously described.
   c. Tritiated Thymidine
      If abnormally low cpm are seen in a sequence of MLC assays, check the shelf life of the tritiated thymidine. The half-life of tritium is 12.3 years and not likely to deteriorate significantly during storage. The thymidine itself, however, has a considerably shorter shelf life and may deteriorate if stored too long. Check the manufacturer's specifications for storing tritiated thymidine.
   d. Culture Medium Evaporation
      Although the culture plates are covered with a plastic lid during incubation, significant evaporation of culture medium from individual wells may occur, especially if there is an air-circulating fan in the incubator. Evaporation results in a loss of growth-supporting medium, and has the effect of making the remaining medium hypertonic, which is detrimental to cell growth. Any empty wells in the culture plate should be filled with medium, PBS or HBSS; this helps to maintain appropriate humidity within the plate and reduce evaporation. In addition, placing the culture plates in a large, covered, ventilated plastic box during incubation allows circulation of humidified 5% CO_2 in air, but reduces the evaporation effect created by the air-circulating fan.
   e. Harvest Machine
      Inappropriately variant replicates or culture combinations that show excessively high cpm can sometimes be traced to a harvest machine that has not been properly cleaned or that is "leaking" radioisotope from one filter disk to another.
   f. Contamination
      Excessively high cpm may be due to contamination of cultures. In this case, responder or stimulator cells cultured in media alone have a high cpm as well. It is highly recommended that steps 3 through 10 of the MLC procedure be performed in a vertical laminar flow hood to minimize the potential for contamination.

3. **Patients**
   The quality and reliability of an MLC reaction is dependent upon the functional integrity (both responding and stimulating capacity) of the cells that are used in the assay. Samples from patients with leukemia, aplastic anemia or renal failure can present several problems.
   a. Leukemia patients
      Abnormal MLC reactions are frequently seen when culturing cells from patients with acute or chronic leukemia. These abnormal reactions are usually seen as significantly elevated cpm in the patient's responding combinations, with consequent loss of discrimination, or as reduced or absent ability of patient cells to stimulate and/or respond. These aberrant reactions may result from a number of factors, including the presence of tumor or other immature cells in the peripheral blood of patients in leukemic relapse,[2,10,12,13] the treatment of the leukemia with lymphocytotoxic drugs or irradiation, or a selective derangement of other cellular elements of the blood by the leukemia. The latter condition can be associated with a generalized loss of immunoregulatory integrity in the patient and/or the occurrence of suppressor cells.[5,6,9,19] Alterations in MLC technique or in the timing of MLC tests that may circumvent such problems include:
      1) Postpone MLC testing of relapse patients, if possible, until a remission has been achieved and the patient has been off chemotherapy for one to two weeks.
      2) Carefully monitor the type of chemotherapy that the patient is currently receiving or has received within the past several weeks. Drugs that are particularly detrimental to lymphocyte function in MLC include cytotoxic drugs (cyclophosphamide [Rx Cytoxan], an alkylating agent), the anthracyclines (Daunomycin

or Adriamycin, DNA crosslinking agents), and enzyme analogues (L-Asparaginase, an antimetabolite). When given in high dosage (usually to patients with chronic myelogenous leukemia [CML] in the accelerated phase of the disease), hydroxyurea, a cytolytic agent which appears to cause immediate cessation of DNA synthesis in susceptible cells, is very detrimental to lymphocyte function. Cells from CML patients in the chronic phase of the disease who are being treated with low (maintenance) doses of hydroxyurea, however, will usually function adequately in MLC.

3) If patient cells do not function as normal stimulators or responders, consider culturing patient cells at multiple concentrations, i.e., 10^4, 5 x 10^4 and 10^5 stimulating and responding cells per culture.
4) To lower the background cpm in patients with CML a higher dose of irradiation (up to 10,000 rad) for stimulator cells is recommended.
5) For acute leukemia patients in relapse or for any patient with chronic myelogenous leukemia, it is advisable to perform an additional lymphocyte purification step after the LSM separation:
   i. T cell rosetting can be performed to obtain a population of functionally normal responder cells.[16] This fraction often will work as a stimulator population as well as a responding population.
   ii. Passage of the patient's mononuclear cell fraction over a nylon wool column and harvesting of adherent cells may be effective in obtaining a population of normal stimulator cells. Cells in the eluate (nonadherent) fraction may serve as relatively normal responder cells.
   iii. A second gradient separation of the patient's mononuclear cell fraction using Percol may be effective in selecting normal stimulator and responder cells.[14]
   iv. The use of Dynabeads for positive or negative selection of normal cells from the patient may be effective.
   v. Monoclonal antibodies and complement may be used to purge the cell suspension of undesirable cells, yielding a population of functionally intact lymphocytes for MLC culturing. See section Lymphocyte Isolation chapter for a discussion of appropriate antibodies and techniques for their use.
   vi. Functional T lymphocytes may also be separated using T Lympho-Kwik isolation medium. If stimulator cells are isolated by B Lympho-Kwik, quite often no irradiation or mitomycin-C treatment is required (for the above isolation methods, see chapter on lymphocyte isolation).

b. Patients with aplastic anemia
Several abnormalities may occur when cells from patients with aplastic anemia are tested in MLC.[19-21] These problems can result from the aplasia itself (loss of stimulating cells, perturbations of lymphocyte and monocyte subpopulations) or to prior treatment (blood transfusions; steroid and ATG therapy).
1) Avoid culturing cells from aplastic patients in autologous plasma, as the plasma may contain inhibitory or enhancing factors that result from blood transfusions.
2) Cells from many severely aplastic patients may show impaired ability, or complete inability, to stimulate in MLC. Increasing the number of patient stimulating cells in selected MLC combinations, as noted for leukemic patients or decreasing the dosage of irradiation (down to 1,000 rad), may be effective in overcoming this problem.
3) Cells from some patients may show heightened response characteristics in MLC, including significantly elevated responses to cells from HLA identical siblings. Reasons for such increased activity are unclear but may relate to derangements of immunoregulatory capacity in certain patients as a result of loss of marrow function, or to sensitization to non-HLA antigens through blood transfusion.

c. Patients with renal failure
Cells from patients suffering from endstage renal disease can display many of the abnormal characteristics that are shown by cells from leukemia or aplastic patients noted above.[7,26] The predominant problem encountered is with the response characteristics of the patient's cells; this is most likely attributable to the renal disease itself or to its treatment. In general, the best method for avoiding such problems is to take a careful patient history and to time the MLC study to coincide with optimal clinical conditions. Consider:
1) Uremia. Cells from uremic patients are known to exhibit reduced levels of reactivity against PHA and allogeneic cells.[18] Although this may be a chronic condition unaffected by a single dialysis treatment, it may be worthwhile to obtain patient blood samples following dialysis in an attempt to reduce the effect of uremic plasma on lymphocyte reactivity. Keep in mind, however, that "nonspecific" activation of patient lymphocytes, manifested as an elevated patient autologous control, may be seen if blood samples are obtained immediately following dialysis.
2) Recent transfusion history. Blood transfusion therapy may affect the behavior of patient leukocytes in culture, leading to reduced reactivity that may be related to the presence of suppressor cells.[4]
3) Chemotherapy. Immunosuppressive therapy with steroids, cyclophosphamide and other antimetabolites (usually administered as treatment for the patient's primary disease), as well as other types of chemotherapy may profoundly affect the in vitro reactivity capacity of patient lymphocytes.

4. **Other Unexpected Results**
a. Strong stimulation (mutual RR >20%) between HLA identical siblings. The most likely explanation is an HLA-B/D-region recombination in one of the individuals being tested. Check family HLA typing, especially DR typing, carefully. Repeat the MLC study using siblings with informative genotypes if possible. Such reactivity may also be the result of parental homozygosity in which siblings that are HLA-A,B locus identical have

inherited different parental haplotypes that differ for HLA-D determinants. The latter can be confirmed by DNA typing of HLA-DR or DQ alleles, thereby defining the subtypes of serologically identical antigens.

b. Weak uni- or bi-directional reactivity between HLA identical siblings. Reactivity of this type is suggestive of disease-related phenomena in the patient. Check family HLA typing and MLC control combinations carefully. As discussed above, cells from patients with leukemia or aplastic anemia may show moderate levels of reactivity with those of a matched sibling, possibly related to chemotherapy, to blood transfusion, or to disease-caused aberrations of lymphoregulatory mechanisms. Unidirectional reactivity is often low-grade and usually does not approach the mean RR value (50%) expected from 1h incompatible family members. Combinations that are truly HLA-D region incompatible should generate bi-directional reactivity in MLC, making unidirectional reactions suspect.

c. Suppressor cells. Unusual or unexpected patterns of in vitro reactivity can result from the activity of suppressor cells, usually of patient origin. The observed effect or suppressor cells can be to decrease stimulation or to decrease ability to respond. For a review of this phenomenon, including culture techniques used to study the effect of suppressor cells, see references 4 and 15.

d. Elevated autologous control ("high background"). This may occur with patients or normal individuals. Each laboratory should define what constitutes an elevated autocontrol and at what CPM levels the controls become unacceptably high. A minimum response criterion might be an SI of >10:1 to reference control cells.
   1) If the elevated control occurs in a patient culture, consider:
      i. contamination of cultures
      ii. remission-relapse status (leukemia)
      iii. patient viral or bacterial infection
      iv. recent transfusion history
   2) If the elevated control occurs in a normal individual, consider:
      i. contamination of cultures
      ii. viral infection (cold, flu)
      iii. other medical factors
      iv. technical factors (serum source, medium, harvest machine, etc.)

e. Backstimulation. The phenomenon of backstimulation, in which inactivated homozygous stimulator cells release blastogenic factors (IL-2?) upon culturing with heterozygous responder cells, has been reported.[25] This problem can usually be overcome by increasing the dose of irradiation that is used to block stimulator cells. It is important to keep in mind that increasing levels of radiation may also decrease the ability of cells to stimulate in MLC.

5. **Common Variations**

The standard MLC technique is amenable to a number of technical variations and modifications. These may be especially useful in tailoring the technique for specific needs or circumstances that arise in the testing of certain types of patients. It is advisable, however, that any modifications of the standard technique be carefully tested in the individual laboratory, subjected to quality control procedures, and an appropriate data base developed with which to compare the changes in MLC results produced by the modifications.

a. Culture plates

The standard MLC test is usually performed in round, or "U", bottom microtiter plates. In some laboratories the use of flat bottom plates, which are available in full (0.32 cm^2) and "half-area" (0.16 cm^2) sizes, is preferred. If flat bottom plates are used, the number of cells cultured per well will likely need to be increased from 5×10^4 to 1×10^5 stimulators and responders.

b. Incubation time

Variation in the total culture time may be useful in some circumstances, especially when testing patients with leukemia or lymphoproliferative disease. Culturing beyond 6 days is not advisable, due to the increasing number of cells that leave "S" phase after hrs 138-144 and are no longer synthesizing DNA. Shorter culture periods (4 to 5 days), however, may be tested as one method of reducing the effect of spontaneously dividing cells that can obscure a discriminative response on day 6. The offsetting cost of a shorter culture period is the lower cpm values that are usually seen.

c. Label time

The amount of time that the dividing cultures need to be labeled with radioisotope is variable and should be assessed in the individual laboratory. In general, the radiolabel may be present in the cultures for 3-18 hrs prior to harvesting: times less than 3 hrs represent a significant thymidine dose limitation and times longer than 18 hrs do not provide a significant incremental advantage in uptake of thymidine. Within these limits variation is possible, and each laboratory should determine an optimal labeling period that gives reproducible results and is consistent with conditions in the laboratory.

d. Anticoagulant

The choice of which anticoagulant to use at the time of sample acquisition is an important issue for each laboratory to address. Probably the most common anticoagulant in current use in MLC testing is heparin, usually available in liquid form as sodium heparin. This is the recommended type of heparin; lithium heparin appears to adversely affect cell viability and quality. If preservative-free heparin is available, it is the anticoagulant of choice; sodium heparin that is preserved with benzoyl alcohol or methylparaben/propylparaben

is acceptable. Green top vacutainer tubes, which contain heparin in crystalline form, are convenient to use but in our experience give variable results in MLC testing. This may be because of variation in the concentration and/or type of heparin in a given tube, or because of different types of preservative that may be present but are difficult to document. Some laboratories report excellent results with these vacutainer tubes.

A second type of anticoagulant is ACD (acid citrate dextrose) or CPD (citrate-phosphate-dextrose), commonly used in blood banking for the collection of whole blood. Each of these represents a suitable alternative to heparin; some laboratories report excellent results with shipped blood that has been drawn into ACD or CPD.

An alternative to the use of anticoagulants is to defibrinate the whole blood immediately after it is drawn. This is accomplished by transferring the whole blood to a flask or tube containing 3-4 mm glass beads and gently rocking the container until clotting has occurred (see chapter on Lymphocyte Isolation). Mononuclear cells obtained from defibrinated blood may display superior response and stimulation characteristics in MLC since they have not been exposed to anticoagulant.

Limitations of Procedure

The MLC assay presents technical challenges to the laboratory, where it may suffer by comparison to other measures for measuring HLA-D region compatibility between recipients and potential marrow donors. It requires a minimum of seven days for completion, making it a time-consuming and costly alternative. Additionally, functionally intact mononuclear cells are needed from both the recipient and donor. Because of the hematopoietic abnormalities often present in the patients being tested in the MLC, there may be significant numbers of failed tests due to uninterpretable responses by the reacting cells.

References

1. Bach FH, Hirschorn K, Lymphocyte interaction: A potential histocompatibility test in vitro. Science 143:813, 1964.
2. Bach ML, Bach FH, Joo P, Leukemia-associated antigens in the mixed leukocyte culture test. Science 166:1520, 1969.
3. Bain B, Vas M, Lowenstein L, The development of large immature mononuclear cells in mixed leukocyte cultures. Blood 23:108, 1964.
4. Bean MA, Mickelson E, Yanagida J, Ishioka S, Brannen GE, Hansen JA, Suppressed antidonor MLC responses in renal transplant candidates conditioned with donor-specific transfusions that carry the recipient's noninherited maternal HLA haplotype. Transplantation 49:382, 1990.
5. Brankovan V, Bean MA, Martin PJ, Hansen JA, Sadamoto K, Takahashi Y, Akiyama M, The cell surface phenotype of a naturally occurring human suppressor T-cell of restricted specificity: Definition by monoclonal antibodies. J Immunol 131:175, 1983
6. Bryan CF, Broxmeyer HE, Hansen J, Pollack M, Dupont B, Identification of an MLC suppressor cell population in acute leukemia. Transplant Proc 10:915, 1978.
7. Daniels JC, Sakai H, Remmers AR Jr, Sarles HE, Fish JC, Cobb EK, Levin WC, Ritzman SE, In vitro reactivity of human lymphocytes in chronic uraemia: analysis and interpretation. Clin Exp Immunol 8:213, 1971.
8. Dupont B, Hansen JA, Yunis EJ, Human mixed-lymphocyte culture reaction: Genetics, specificity and biological implications. In: *Advances in immunology*, Academic Press, New York, p. 107, 1976.
9. Engleman EG, McDevitt HO, A suppressor T-cell of the mixed lymphocyte reaction specific for the HLA-D region in man. J Clin Invest 61:828, 1978.
10. Fefer A, Mickelson E, Thomas ED, Leukaemia antigens: Stimulation of lymphocytes in mixed culture by cells from HLA identical siblings. Clin Exp Immunol 23:214, 1976.
11. Fehrman I, Ringden O, Lymphocytes from multitransfused uremic patients have poor MLC reactivity. Tissue Antigens 17:386, 1981.
12. Fridman WH, Kourilisky FM, Stimulation of lymphocytes by autologous leukaemic cells in acute leukaemia. Nature 224:277, 1969.
13. Gutterman JU, Rossen RD, Butler WT, McCredie KB, Bodey GP, Freireich DJ, Hersh EM, Immunoglobulin on tumor cells and tumor-induced lymphocyte blastogenesis in human acute leukemia. N Engl J Med 288:169, 1973.
14. Hakos G, Rayment C, Honeyman M, Bashir H, Percoll separation of leukemic leukocytes for MLC matching prior to bone marrow transplantation. Transplantation 39:323, 1985.
15. Hutchinson IV, Suppressor T cells in allogeneic models. Transplantation 41:547, 1986.
16. Kaplan ME, Clark C, An improved rosetting assay for detection of human T lymphocytes. J Immnunol Meth 5:131, 1974.
17. Klatzmann D, Gluckman JC, Foucault C, Bensussan A, Assobga U, Duboust A, Suppression of lymphocyte reactivity by blood transfusions in uremic patients. Transplantation 35:332, 1983.
18. Kunori T, Fehman I, Ringden O, Moller E, In vitro characterization of immunological responsiveness in uremic patients. Nephron 26:234, 1980.
19. McMichael AJ, Sasazuki T, A suppressor T-cell in the human mixed lymphocyte reaction. J Exp Med 146:368, 1977.
20. Mendel NR, Guppy D, Bodmer WF, Festenstein H: Joint report: Data management and assignment of scores to MLC data. In: *Histocompatibility Testing*, 1977. WF Bodmer, JR Batchelor, JG Bodmer, H Festenstein, PJ Morris, eds. Munksgaard, Copenhagen, p. 90, 1977.
21. Mickelson EM, Fefer A, Thomas ED, Aplastic anemia: Failure of patient leukocytes to stimulate allogeneic cells in mixed leukocyte culture. Blood 47:793, 1976.

22. Mickelson EM, Fefer A, Storb R, Thomas ED, Correlation of the relative response index with marrow graft rejection in patients with aplastic anemia. Transplantation 22:294, 1976.
23. Mickelson EM, Beatty PG, Storb R, Hansen JA, Immune responses in an untransfused patient with aplastic anemia: Analysis of cytolytic and proliferative T cell clones. Human Immunology 10:189, 1984.
24. Ollier W, Mendell N, Sachs J, Jaraquemada D, Evans S, Pegrum G, Festenstein H, Sources of variance in the double normalized value: an evaluation of its reproducibility as a measure on HLA-D locus identity. Tissue antigens 18:141, 1981.
25. Sasazuki T, Mcmichael A, Radvany R, Payne R, McDevitt H, Use of high dose x-irradiation to block back stimulation in the MLC reaction. Tissue Antigens 7:91, 1976.
26. Sengar DPS, Opelz G, Terasaki PI, Suppression of mixed leukocyte response by plasma from hemodialysis patients. Tissue Antigens 3:22, 1973.
27. Thorsby E, du Bois R, Bondevik H, Dupont B, Eijsvoogel V, Hansen JA, Jersild C, Jorgensen F, Kissmeyer-Nielsen F, Lamm LU, Schellekens PThA, Svejgaard A, Thomsen M, Joint report from a mixed lymphocyte culture workshop. Tissue Antigens 4:507, 1974.
28. Thorsby E, Piazza A: Joint report from the sixth international histocompatibility workshop conference. II. Typing for HLA-D (LD-1 or MLC) determinants. In: *Histocompatibility Testing*, 1975, F Kissmeyer-Nielsen, ed. Munksgaard, Copenhagen, p. 414, 1975.
29. Yunis EJ, Amos DB, Three closely linked genetic systems relevant to transplantation. Proc Natl Acad Sci USA 68:3031, 1971.

HLA-Dw Typing

Nancy Reinsmoen and Eric Mickelson

Purpose

With the recent application of DNA methodologies for typing HLA class II specificities, the homozygous typing cells (HTC) approach to typing for HLA-D region identity is no longer commonly used. However, in the context of allotransplantation the technique may be useful in identifying acceptable mismatches, i.e., identifying those HLA molecules of the donor and recipient which may differ by one or more amino acids but which cannot be discriminated by T cells. In addition, this technique may be useful as a measurement of the change of an immune response with time. For example, the development of donor antigen-specific hyporeactivity has been assessed posttransplant in kidney transplant recipients by measuring the change in response to HTCs defining the donor's HLA-Dw specificities. The purpose of this chapter is to present the Dw typing technique in the context of current usage in the clinical laboratory and to provide a historical review of the basis for assigning the HLA-Dw specificities.

Theoretically, cells from individuals who are homozygous for HLA-D region determinants can be used as typing reagents (stimulator cells) in a mixed lymphocyte reaction to identify responder cells possessing the HLA-Dw specificity for which the HTC is homozygous. Responder cells sharing HLA-D region determinants with a given HTC would be expected to generate very weak mixed lymphocyte culture (MLC) reactivity compared to those responder cells that do not.[11,21].

The technique used for HLA-Dw typing is basically that used for the standard MLC, except that HTCs are used as stimulators and cells of undefined Dw specificity are used as responders. HTCs are chosen as typing reagents if: (1) they do not stimulate a significant response in appropriate combinations within the family from which they were derived; (2) they do not stimulate (or stimulate only weakly) cells of other HTCs that are used to define the same Dw specificity; and (3) they can be used successfully to "type" an unrelated panel, i.e., to distinguish between cells that respond strongly and those that respond weakly. Cells showing a weak (i.e., "typing") response are assumed to express the specificity that is defined by the particular HTC. This methodology is relatively straightforward and has been thoroughly reviewed in this manual (see the MLC chapter) and other publications.[26,33,10,18]

The concept of using homozygous cells in the quantitation of the MLC was first described in the pig[3] and subsequently was adapted for use as a cellular typing method in humans.[11,21,48,7,24] Most of the initial studies involving HLA-D homozygous cells utilized lymphocytes from offspring of first cousin marriages who had inherited two haplotypes that were identical by descent.[21,48] Subsequently, HTCs were identified in the random (outbred) population and were submitted to several International Histocompatibility Workshops (IHW). Studies from these workshops allowed the definition of 23 HTC-defined HLA-Dw specificities (HLA-Dw1-Dw23) and three specificities defined by cloned T cells (HLA-Dw24-26) which identify subgroups of the HLA-DR52 specificity (Table1).

Although the technique for typing with HTC is technically relatively simple, analysis of the resulting data, assignment of an HLA-Dw specificity, and the interpretation of results can be difficult. The results of assays utilizing homozygous typing cells are dependent upon a large number of factors, including the number of individual antigenic determinants involved in MLC stimulation, the ability of a given responder cell to respond, the inherent stimulatory capacity of a given HTC (independent of the HLA-D region antigens it expresses), the production of helper and suppressor factors during culture, and technical variation. In practice, therefore, few HTCs demonstrate clear bimodal distribution patterns of "typing" (weak) vs. "non-typing" (strong) responses; frequently questionable or borderline typing responses are observed.

The HTC-defined HLA-Dw specificities (Dw1-w23) represent clusters of antigenic determinants predominantly associated with class II molecules. In certain combinations, class I molecules can also stimulate a weak T lymphocyte proliferation. The response to a given HTC represents the aggregate reactions of multiple responding clones recognizing determinants associated with DR, DQ and DP molecules expressed by the stimulating HTC. The antigenic products of HLA-DP genes are not felt to generate strong proliferative responses in primary MLC; DR and DQ antigens appear to predominate. However, since the HLA-DP genes are not in strong linkage disequilibrium with the DR and DQ genes of a given haplotype, the weak stimulation generated by these antigens tends to obscure a clear bimodal response pattern and make the assignment of a Dw specificity more difficult. There is sufficient linkage disequilibrium between certain DR and DQ alleles to generate HLA-Dw "haplotypes." Cells from individuals who have inherited two similar parental Dw haplotypes will behave as functionally homozygous stimulators in MLC and identify responder cells that possess the relevant Dw phenotypes.

HLA-Dw specificity clusters defined with HTCs identify subgroups of the serologically-defined DR antigens (i.e., Dw clusters are subtypic to DR antigens). HTC-defined Dw specificities are shown in Table 1. T cell clonal analysis has provided evidence that both DR products as well as DQ products can stimulate T lymphocytes; however, the contribution of the stimulatory determinants associated with these products appears to differ for various Dw haplotypes. For example, DQ products appear to play an important role in the definition of the DR2-associated Dw specificities and in the Dw11 vs. Dw17 specificities, but less of a role in other haplotypes.[2] Cloned T cell reagents submitted to the Tenth International Histocompatibility Workshop identified three cellularly-defined subgroups of the serologically-defined HLA-DR52 specificity: Dw24, Dw25 and Dw26. These DR52 subgroups of the DRB3-encoded molecule have been shown to be associated with several distinct DR haplotypes (Table 1).

Table 1. HLA-Dw Specificities, North American Population

HLA-DR	HLA-Dw	HLA-DRB1	HLA-DR 52/53, Dw 24-26[b]	HLA-DQ	HLA-B	Racial/ Ethnic	Gene Frequency[a]	References
HLA-DR1	Dw1	0101		DQ5	B35, B27		0.0898	6, 44
	Dw20	0102		DQ5	B14	Jewish	0.0034	
	blank						0.0108	
HLA-DR2 DR15	Dw2	1501		DQ6	B7		0.1642	5, 8, 12
DR15	Dw12	1502		DQ1	B52	Oriental	0.0068	23, 25
DR16	Dw21	1601		DQ5			0.0114	36, 37
DR16	Dw22	1602		DQ7		Am Indian		38, 41
	blank						0.0148	49
HLA-DR3 DR17	Dw3	0301	DR52a, Dw24	DQ2	B8		0.1150	19
DR17	Dw3 var.	0301	DR52b, Dw25	DQ2	B18			
DR18	Dw new	0302	DR52a, Dw24	DQ4	B42	Black	0.0023	
	blank						0.0125	
HLA-DR4	Dw4	0401	DR53	DQ7	B44(12)		0.0910	4, 16
				DQ8	B62(15)			25, 30
	Dw10	0402	DR53	DQ8	B38	Jewish	0.0142	32, 35
	Dw13	0407/0403	DR53	DQ3			0.0136	
	Dw14	0404/0408	DR53	DQ8	B60(40)		0.0545	
	Dw15	0405	DR53	DQ4		Oriental		
	blank						0.0263	
HLA-DR5 DR11	Dw5	1101/1104	DR52b, Dw25	DQ7			0.0665	9, 20
DR11	DB2		DR52b, Dw25	DQ1				
DR12	DB6	1201	DR52b, Dw25	DQ7			0.0011	
	blank						0.0344	
HLA-DR6 DR13	Dw18	1301	DR52a, Dw25	DQ6			0.0928	14, 15
DR13	Dw18	1301	DR52b, Dw25	DQ6				17, 34
DR13	Dw19	1302	DR52c, Dw26	DQ1				46, 47
DR14	Dw9	1401	DR52b, Dw25	DQ5			0.0165	
DR14	Dw16	1402	DR52a, Dw24	DQ7	B62	Am Indian	0.0006	
	blank						0.0292	
HLA-DR7	Dw7	0701	DR53	DQ2			0.1207	9, 20
	Dw17	0701	DR53	DQ2	B44			22
	Dw11	0701		DQ9	B57			
	DB1	0701	DR53	DQ2	B13			
	blank						0.0102	
HLA-DR8	Dw8.1	0801		DQ4		Caucasian	0.0211	29
	Dw8.2	0802		DQ4		Am Indian		
	Dw8.3	0803		DQ1		Oriental		
	Dw new			DQ7		Dutch		
	blank						0.0074	
HLA-DR9	Dw23	0901	DRw53	DQ9		Oriental	0.0053	28, 31
	blank						0.0011	
HLA-DR10	Dw new	1001		DQ5		Jewish	0.0028	1, 13
	blank					French	0.0017	

a. HLA-Dw Gene Frequencies (1988) North American Caucasian Population.
The data represent combined results from the University of Minnesota, Minneapolis, MN and Fred Hutchinson Cancer Research Center, Seattle, WA, testing 886 individuals over a 10 year period. The Dw6 and Dw7 subgrouping data was not available for all of the early typing; however, more recent results indicate the following percentages: in 102 DRw6 haplotypes tested, 9% typed as Dw6, 49% as Dw18 and 42% as Dw19. In 63 DR7 haplotypes tested, 6% typed as Dw7, 67% as Dw17, and 27% as Dw11.

b. References for Dw24-26 subgroups: 19, 27, 43, and 45.

Specimen

1. Peripheral blood lymphocytes obtained in heparin or ACD
2. Tissue-infiltrating T cells propagated from biopsy or obtained by mechanical or enzymatic digestion of tissue

The following specimens are unacceptable:

1. Clotted blood
2. Specimens more than three days old

Reagents

The reagents are the same as those used for the MLC technique (see MLC chapter II.C.1).

Instrumentation

Same as those used for the MLC technique (see MLC chapter II.C.1).

Procedure

The HLA-D typing technique utilizes the basic MLC procedure, incorporating HTC of well-defined specificity as stimulator cells. HLA-D typing assays are set up using frozen stimulator and responder cells. A typical assay includes 24 responder cells, 3-4 HTCs per Dw specificity, and pooled stimulating cells (three unrelated cells per pool, selected to include no duplication of Dw/DR specificities).

1. Thaw cells according to standard procedure.
2. Use the autologous response, or responding cells or stimulator cells cultured alone in 20% PHS-RPMI, as negative controls.
3. Perform cell viabilities before plating. Irradiate stimulating cells at 3000 rads (^{137}Cs irradiator). Plate HLA-D typing experiments in round bottom microtiter plates. Pipette 50,000 responding cells and 50,000 stimulating cells in a total volume of 0.2 ml in each well.
4. Incubate plates in a humidified 37°C, 5% CO_2 incubator for 5 days.
5. Label with tritiated thymidine (1.0 µCi/well, 6.7 Ci/mM specific activity) for 18 hr.
6. Harvest cultures according to standard MLC procedure and count DNA residue in a scintillation counter.

Calculations

Responder normalized values (RNV) and double normalized values (DNV) are calculated according to the method of Ryder et al. (1975) in the Sixth International Histocompatibility Workshop (IHW). The individual responses to stimulating HTC are normalized by dividing the median cpm of the test (responding cell) value by the 75th percentile ranked response of all responding cell median cpm and multiplying each result by 100 to produce an RNV. Double normalized values are obtained by ranking the resulting RNV for each stimulating cell, dividing each RNV by the 75th percentile value and multiplying by 100, as follows:

Responder Normalized Values (RNV):

75th ranked response (cpm): The individual responses to each stimulating HTC for a given responder cell are ranked from lowest to highest; the 75th % highest response is designated as the 100% reference response.

Test (cpm): response to given stimulating HTC

$$RNV = \frac{test\ (cpm)}{75th\ ranked\ response\ (cpm)}\ x\ 100$$

Double Normalized Values (DNV):

75th ranked RNV: All stimulation values for a given stimulator cell are ranked lowest to highest; the 75th % highest RNV is designated as the 100% reference stimulation value.

Test RNV: Individual RNV responses to stimulation by a given stimulator cell.

$$DNV = \frac{test\ RNV}{75th\ ranked\ RNV}\ x\ 100$$

Results

The typing responses are assigned by interpretation of the DNV values as follows:

A "positive" typing response (TR) is assigned if:

a) the responses to the majority of the HTCs defining a given specificity are ≤29 DNV, or
b) responses of ≤50 DNV to the majority of HTCs of a given Dw specificity are reproducible in repeated testings.

A "possible" typing response is assigned if:

a) the responses to the majority of the HTCs defining a given specificity are between 29 and 50 DNV for a single testing, or
b) responses between 29 and 50 DNV for at least two HTCs per specificity are reproducible in repeated testings.

No typing response is assigned if all responses are > 50 DNV.

An alternative method of DNV scoring and antigen assignment is that used in the 8th IHW (Dupont et al., 1980). The DNV calculations normalize the different responding and stimulating capabilities of each cell tested. The raw data are

thus converted to a normalized value (the DNV) which can be used to compare typing responses within one experiment or among several experiments. The DNV values should be relatively small (≤35%) for responder cells that share the Dw specificity of the relevant HTC, and relatively larger (>35%) for responder cells that do not. Optimally there should be a bimodal distribution of responses in a given experiment, with clear separation between cells that are positive for a given specificity and those that are negative. In actual practice, many HTC typing profiles do not show clear bimodal distribution, presumably because of several factors: multiple class II stimulatory determinants on a given HTC which may be expressed at different levels of relative density; DP disparity between responder cell and the HTC; immunoregulatory factors affecting the stimulating and responding capacity of the cultured cells; and technical factors in the assay itself. Since a responder cell that is positive for a given Dw specificity may not generate a low typing response to each HTC of that specificity, it is necessary to use several HTCs per Dw specificity.

Procedure Notes

The current use of HLA-Dw typing in the context of the clinical laboratory has changed with the advent of DNA typing for HLA polymorphisms. Although DNA technologies provide more exact information regarding the HLA class II polymorphisms, it remains to be determined whether cellular assays such as the MLC and HLA-Dw typing can provide information concerning acceptable degrees of HLA mismatching between donor and recipient, that is, structural polymorphisms that may not be recognized as functionally different by effector T cells.

HLA-Dw typing and the MLC techniques are being used currently to investigate the development of donor antigen-specific hyporeactivity following renal transplantation.[39] These assays may be useful in identifying those patients who are good candidates for withdrawal or tapering of immunosuppressive therapy based on their apparent successful immunoregulation of response to disparate (donor) antigens. The development of donor antigen-specific hyporeactivity as measured by MLC and HLA-Dw typing assays correlates with improved late renal transplant outcome as evidenced by fewer late rejection episodes, a lower incidence of chronic rejection and fewer graft losses.[40]

In conclusion, the HLA-Dw typing technique described in this chapter has been used historically to determine HLA-D region compatibility. This technique remains useful for assessing T cell epitopes and for investigating immune regulation.

References

1. Amar A, Mickelson E, Hansen JA, Shalev Y, Brautbar C, HLA-Dw "SHY": A new lymphocyte defined specificity associated with HLA-DRw10. Hum Immunol 11:143, 1984.
2. Bach FH, Reinsmoen N, Segall M, Definition of HLA antigens with cellular reactants. Transplant Proc 15:102, 1983.
3. Bradley BA, Edwards JM, Dunn DC, Caine RY, Quantitation of mixed lymphocyte reaction by gene dosage phenomenon. Nature New Biol 240:54, 1972.
4. Cairns S, Curtsinger JM, Dahl CA, Freeman S, Alter BJ, Bach FH, Sequence polymorphism of HLA-DRß1 alleles relating to T cell-recognized determinants. Nature 317:166, 1985.
5. Cohen N, Amar A, Oksenberg J, Brautbar C, HLA-D clusters associated with DR2 and the definition of HLA-D "AZH", a new DR2 related HLA-D specificity in Israel. Tissue Antigens 24:1, 1984.
6. Cohen N, Friedmann S, Szafer F, Amar A, Cohen D, Brautbar C, Polymorphism of the HLA-DR1 haplotype in the Israeli population investigated at the serological, cellular and genomic levels. Immunogenetics 23:252, 1986.
7. Dausset J, Sasportes M, Lebrun A, Mixed lymphocyte culture (MLC) between HLA-A serologically identical parent-child and between HLA homo and heterozygous individuals. Transplant Proc 5:1511, 1973.
8. DeMarchi M, Varetto O, Savina C, Borelli I, Curtoni ES, Carbonara AO, Relationships between HLA-D and DR. In: *Histocompatibility Testing 1980*; PI Terasaki, ed., Los Angeles; p. 893, 1980.
9. Dupont B, Braun DW, Yunis EJ, Carpenter CB, Joint report: HLA-D by cellular typing. In: *Histocompatibility Testing 1980*; PI Terasaki, ed.; Los Angeles; p. 229, 1980.
10. Dupont B, Hansen JA, Yunis EJ, Human mixed lymphocyte culture reaction: Genetics, specificity, and biological implications. Adv Immunol 23:107, 1976.
11. Dupont B, Jersild C, Hansen GS, Nielsen S, Thomsen M, Svejgaard A, Typing for MLC determinants by means of LD-homozygous and LD-heterozygous test cells. Transplant Proc 5:1543, 1973.
12. Freidel AC, Betuel H, Gebuhrer L, Farre A, Lambert J, Distinct subtypes of HLA-D associated with DR2. In: *Ninth International Workshop and Conference Newsletter No. VIII*: 24, 1986.
13. Gebuhrer L, Betuel H, Lambert J, Freidel AC, Farre A, Definition of HLA-DRw10. In: *Newsletter No. VII, Ninth International Histocompatibility Workshop and Conference*; p. 41, 1984.
14. Gorski J, Mach B, Polymorphism of human Ia antigens: Gene conversion between two DR ß loci results in a new HLA-D/DR specificity. Nature 322:67, 1986.
15. Gorski J, Tilanus M, Giphart M, Mach B, Oligonucleotide genotyping shows that alleles at the HLA-DRßIII locus of the DRw52 supertypic group segregate independently of known DR or Dw specificities. Immunogenetics 25: 79, 1987.
16. Groner J, Watson A, Bach FH, Dw/LD related molecular polymorphism of DR4 beta chains. J Exp Med 157:1687, 1983.
17. Grosse-Wilde H, Doxiadis I, Brandt H, Definition of HLA-D with HTC. In: *Histocompatibility Testing 1984*; ED Albert, ed.; Springer-Verlag, Berlin; p. 249, 1984.
18. Hartzman RJ, Segall M, Bach FH, Histocompatibility matching. VI. Miniaturization of the mixed leukocyte test. A preliminary report. Transplantation 11: 268, 1971.

19. Irle C, Jaques D, Tiercy JM, Fuggle SV, Gorski J, Termijtelen A, Jeannet M, Mach B, Functional polymorphism of each of the two HLA-DRß chain loci demonstrated with antigen-specific DR3- and DRw52-restricted T cell clones. J Exp Med 167:855, 1988.
20. Jakobsen BK, Platz P, Ryder LP, Svejgaard A, A new homozygous typing cell with HLA-D "H" (DB6) specificity. Tissue Antigens 27:396, 1986.
21. Jorgensen F, Lamm L, Kissmeyer-Nielsen F, Mixed lymphocyte cultures with inbred individuals: An approach to MLC typing. Tissue Antigens 4:323, 1973.
22. Karr RW, Immunochemical analysis of the Ia polymorphisms among the family of DR7-associated HLA-D specificities. J Immunol 136:999, 1986.
23. Layrisse Z, Simoney N, Park MS, Terasaki PI, HLA-D and DRw determinants in an American indigenous isolate. Transplant Proc 11:1788, 1979.
24. Mempel W, Grosse-Wilde H, Baumann P, Netzel B, Albert ED, Population genetics of the MLC response: Typing for MLC determinants using homozygous and heterozygous reference cells. Transplant Proc 5:1529, 1973.
25. Mickelson E, Brautbar C, Nisperos B, Cohen N, Amar A, Kim S, Lanier A, Hansen JA, HLA-DR2 and DR4 further defined by two new HLA-D specificities (HTC) derived from Israeli Jewish donors: Comparative study in Caucasian, Korean, Eskimo, and Israeli populations. Tissue Antigens 24:197, 1984.
26. Mickelson E, Hansen J, The mixed lymphocyte culture (MLC) reaction, and typing for HLA-D determinants. In: *AACHT Laboratory Manual*; A Zachary, W Braun, eds.; American Association for Clinical Histocompatibility Testing, New York; p. IV.1, 1981.
27. Mickelson E, Masewicz SA, Cotner T, Hansen JA, Variants of HLA-DRw52 and defined by T lymphocyte clones. Human Immunol 22:263, 1988.
28. Mickelson EM, Nisperos B, Thomas ED, Hansen JA, Definition of LD "4x7": A unique HLA-D specificity defined by two homozygous typing cells. Hum Immunol 4:79, 1982.
29. Mickelson EM, Nisperos B, Layrisse Z, Kim SJ, Thomas ED, Hansen JA, Analysis of the HLA-DRw8 haplotype: Recognition by HTC typing of three distinct antigen complexes in Caucasians, Native Americans and Orientals. Immunogenetics 17:399, 1983.
30. Nepom BS, Nepom GT, Mickelson E, Antonelli P, Hansen JA, Electrophoretic analysis of human "Ia-like" antigens from HLA-DR4 homozygous cell lines: Correlation between ß chain diversity and HLA-D. Proc Natl Acad Sci USA 80:6962, 1983.
31. Nose Y, Matsuoke ES, Tsuji K, A new HLA-D specificity (DKy: homozygous for DRw9) found in the Japanese. Tissue Antigens 18:69, 1981.
32. Nose Y, Sato K, Nakagawa S, Kondok K, Inouye H, Tsuji K, HLA-D clusters associated with DR4 in the Japanese population. Hum Immunol 5:199, 1982.
33. O'Leary J, Reinsmoen N, Yunis E, Mixed lymphocyte reaction. In: *Manual of Clinical Immunology*; American Society for Microbiology, Washington, DC; p. 820, 1976.
34. Ollier W, Doxiadis I, Jaraquemada D, Okoye R, Grosse-Wilde H, Festenstein H, First level testing of HLA-D "blank" HTC. In: *Histocompatibility Testing 1984*; ED Albert, ed.; Springer-Verlag, Berlin; p. 281, 1984.
35. Reinsmoen NL, Bach FH, Five HLA-D clusters associated with HLA-DR4. Hum Immunol 4:249, 1982.
36. Reinsmoen NL, Bach FH, Clonal analysis of HLA-DR and -DQ associated determinants – their contribution to Dw specificities. Hum Immunol 16:329, 1986.
37. Reinsmoen NL, Bach FH, T cell clonal analysis of HLA-DR2 haplotypes. Hum Immunol 20:13, 1987.
38. Reinsmoen NL, Layrisse, Betuel H, Bach FH, A study of HLA-DR2 associated HLA Dw/LD specificities. Hum Immunol 11:105, 1984.
39. Reinsmoen NL, Kaufman D, Matas A, Sutherland DER, Najarian JS, Bach FH, A new in vitro approach to determine acquired tolerance in long-term kidney allograft recipients. Transplantation 50:783, 1990.
40. Reinsmoen NL, Matas AJ, Improved late renal transplant outcome correlates with the development of in vitro donor antigen-specific hyporeactivity. Transplantation (in press).
41. Richiardi P, Belvidere M, Borelli I, DeMarchi M, Curtoni EM, Split of HLA-D and DRw2 into subtypic specificities closely correlated to two HLA-D products. Immunogenetics 7:57, 1978.
42. Ryder LP, Thomsen M, Platz P, Svejgaard A, Data reduction in LD- typing. In: F. Kissmyer-Nielsen, ed: *Histocompatibility Testing 1975*; Munksgaard, Copenhagen; p. 557, 1975.
43. Sheehy MJ, Rowe JR, Konig F, Jorgensen L, Functional polymorphism of the HLA-DR Beta III chain. Hum Immunol 21:49, 1988.
44. Suciu-Foca N, Godfrey M, Kahn R, Woodward K, Rohowsky C, Reed E, Hardy M, Reemtsma K, New HLA-D alleles associated with DR1 and DR2. Tissue Antigens 17:294, 1981.
45. Termijtelen A, van den Berge SJ, van Rood JJ, LB-Q1 and LB-Q2: Two determinants defined in the primed lymphocyte test and independent of HLA-D/DR, MB/LB-E or SB. Hum Immun 8:11, 1983.
46. Termijtelen A, Schreuder GMT, Mickelson EM, van Rood JJ, Ninth International Histocompatibility preworkshop testing of Dw6 HTCs. Two subtypes of Dw6. Tissue antigens 24:10, 1984.
47. Todd JA, Bell JI, McDevitt HO, HLA-DQß gene contributes to susceptibility and resistance to insulin-independent diabetes mellitus. Nature 329:599, 1987.
48. Van den Tweel JG, Bluse van Oud Alblas A, Keuning JJ, Goulmy E, Termijtelen A, Bach ML, van Rood JJ, Typing for MLC (LD) I. Lymphocytes from cousin marriage offspring as typing cells. Transplant Proc 5:1535, 1973.
49. Wu S, Saunders T, Bach FH, Polymorphism of human Ia antigens generated by reciprocal intergenic exchange between two DRß loci. Nature 324:599, 1987.

The Primed Lymphocyte Test (PLT)

Nancy L. Reinsmoen

Purpose

The purpose of this chapter is to provide an overview of primed lymphocyte test (PLT) methodology, theory and principle, as well as the current and future uses of the PLT in histocompatibility testing and in the assessment of alloreactivity. The chapter on T cell cloning will address the expansion of the primed reagents through cloning methodologies and the finer definition of T cell-recognized epitopes that are possible with this approach.

The PLT is a method used to detect the lymphocyte-defined (LD) determinants associated with the MHC antigens by the generation of highly specific reagents. The principle of the PLT technique is to generate responder cells primed against disparities expressed by a stimulator cell by incubating the cells together for a period of 10 days. The primed cells, presumably memory cells, will respond in an accelerated, i.e., secondary, fashion when restimulated by cells from the original stimulator or by other cells which share stimulatory determinants with the sensitizing cell.

Introduction

The *in vivo* generation of lymphocytes capable of responding in an accelerated secondary manner and mediating cytotoxicity was first described in the mouse system.[1] The ability to obtain a secondary proliferative response was demonstrated shortly thereafter in man.[11,33] The PLT studies of Sheehy et al.,[33,34] Bach et al.[5] and Mawas et al.[15] demonstrated that the secondary proliferative response could be used to define determinants of the HLA-D region. Several investigators[2,15,35,37] generated highly discriminatory reagents against HLA-D region determinants by utilizing homozygous typing cells (HTCs) or heterozygous cells which shared one Dw specificity. Initially, HLA-DR as defined serologically was thought to be the major stimulus in the PLT.[7,12,17,36] Subsequently, PLT reagents were identified which were capable of discriminating subgroups of the serologically-defined DR determinants as defined by HLA-Dw typing.[2,7,14,15,17,27,29,34-37] Stimulatory determinants in PLT, which presumably reflect those determinants capable of stimulation in the primary mixed lymphocyte culture (MLC), have been reported to be associated with DR, DQ, and DP loci, the HLA-A, B chromosomal segment,[4,8-10,18,20-22,41,43] as well as determinants not linked to HLA.[25,38,39]

Shaw et. al.[30-32] characterized a new allelic series which they designated SB (secondary B cell alloantigen), now termed DP (Ninth International Workshop 1984), by using unrelated cells phenotypically identical for HLA-A, -B, -C, -DR, -Dw and -DQ specificities as priming cells in the PLT assay. Shaw generated reagents which defined five antigens of a single segregant series mapped between DR and glyoxalase.[3,30] These determinants elicited a weak primary, but strong secondary, MLC response. The weaker primary MLC reactivity elicited by DP molecules may be due to a lower quantity and/or immunogenicity of DP molecules relative to other stimulatory molecules (i.e., DR); however, through clonal expansion of a DP reactive cell, a strong secondary response is observed. Six DP specificities are identified by World Health Organization (WHO) nomenclature, although 38 DPB genes have been identified. The expansion and cloning of primed T cells has provided valuable reagents which identify the cellularly-defined determinants/epitopes associated with the MHC molecule and has expanded our understanding of the allogeneic response.

Currently, in the clinical histocompatibility laboratory setting the PLT can be used in monitoring transplant recipients to assess if primed cells reactive to donor antigens are present in the allograft or at the site of a lesion. The PLT can also be used in the investigation of anomalous MLC reactivity. When the PLT is used in this manner to assess the alloproliferative response, all disparate molecules can potentially elicit a response. T cell cloning may be necessary to differentiate the response to the individual HLA molecules.

Specimen

Fresh or frozen peripheral blood mononuclear cells (PBMC), lymphoblastoid cell lines or graft infiltrating cells can be used in the PLT assay. As with all cellular procedures, care must be taken throughout the procedure to ensure a sterile specimen is obtained. The PBMC specimen may be saved overnight but should be processed within 24 hours of phlebotomy. The specimen should be maintained at room temperature even if being shipped by overnight carrier. Poor cell yields may result from either too cold or too warm temperature conditions. If a patient is receiving one of the following drugs, the proliferative response may be compromised: prednisone, myleran, hydroxyurea, cytoxan, daunomycin, or L-asparaginase. Cells to be used as responder cells in the cell cultures can be frozen prior to use. However, better viability and cell recovery are experienced if the cells are rate frozen and stored in the vapor phase of a liquid nitrogen storage unit.

Unacceptable Specimens

Specimens are considered unacceptable if they are: unsterile, over 24 hrs old, drawn in lithium heparin, clotted or unlabelled.

Instrumentation

There are a number of different harvesting machines and counting systems available, ranging from harvesting the cells on to filter disc sheets, counting the samples in vials or cassettes, or counting directly without the need for scintillation fluid. Consult the manufacturer's instruction manual for the appropriate procedures to follow.

Reagents

1. Culture medium
   RPMI 1640 w/HEPES supplemented with:
   Penicillin-Streptomycin (10,000 units/ml)
   L-glutamine
   Gentamicin (50 mg/ml)
   Prepare and filter through a 0.45 µ filter

For expanding primed reagents, 20% T cell growth factor (TCGF) (Biotest Diagnostics Corp.) or a source of recombinant rIL-2 must be added to the culture medium.

2. ^{3}H-thymidine (^{3}H-Tdr)
   For PLTs and cloning procedures, thymidine (specific activity = 20 Ci/mM) is used at a concentration of 2 µCi/well (40 µCi/ml). Alternatively, thymidine with a specific activity of 6.7 Ci/mM can be used.
3. Pooled human sera (PHS)
   The PHS should be screened in the PLT assay prior to use, according to the following protocol:
   a. Use the normal PLT protocol, with a pool-primed PLT as the responder and cells from three unrelated individuals as stimulator cells.
   b. Test each serum at a 10% final culture concentration in all three responder/stimulator combinations. Use previous serum pool as control.
   c. Perform PLT assay as described below.
   d. Determine if the PHS adequately supports proliferative reactivity.
4. Heat inactivated pooled human sera (iPHS)
   Inactivate PHS by placing it in 56° C waterbath for 30 min.

Procedure

Priming Cells

1. Obtain mononuclear cells by density gradient centrifugation of heparinized peripheral blood. Alternatively, controlled-rate frozen cells may be used for the priming and restimulation protocols.
2. Culture 10 x 10^{6} responding cells in a 50 ml tissue culture flask with 10 x 10^{6} stimulating cells which have been irradiated (3,000 rads).
3. Adjust the final volume to 15 ml with RPMI 1640 containing 10% PHS.
4. Incubate in a humidified 37° C, 5% CO_2 incubator for 10-12 days. On days 2, 4, 6, and 8, add 2 ml RPMI 1640 containing 10% iPHS.
5. After 10-12 days, transfer the primed cells to sterile tubes and wash twice with RPMI 1640. The primed cells may then be either frozen for future use or used immediately in the restimulation assay.

Restimulation of Cells

1. Adjust primed (responder) cells to four cell concentrations: 1, 0.5, 0.25 and 0.125 x 10^{5} cells/ml. If this assay system is used to test cloned cells, concentrations of 0.5 – 1.0 x 10^{5} responder cells/ml are usually used.
2. Irradiate secondary stimulating PBLs at 3000 rads and adjust to 0.5 x 10^{6} cells/ml. Secondary stimulators should include:
   a. cells from the original responding cell in the priming reaction (autologous or negative control)
   b. cells from the original stimulating cell in the priming reaction (reference cell)
   c. various other test cells
3. Add 100 µl stimulating cells and 100 µl primed (responder) cells per well in U bottom microtiter plates. Thus, the final concentration is 50,000 stimulating cells per well and either 10,000, 5,000, 2,500, or 1,250 primed cells per well.
4. Incubate cultures for 48 hr in a humidified 37° C, 5% CO_2 incubator.
5. Add 50 µl/well of ^{3}H-Tdr at 40 µCi/ml (2 µCi/well) and incubate for 18 hr.
6. Terminate cultures by immediately harvesting or by placing plates, covered with a pressure-sensitive adhesive film, in a 4° C refrigerator until harvested.
7. Harvest cultures and count in a scintillation counter.

Expansion of Primed Cells

1. Prepare feeder cells.
   The choice of feeder cells depends upon the specificity and use of the reagent, and must be left to the discretion of the investigator. Three suggested feeder cell combinations are described below.
   a. PBL: Irradiate (6000 rads) PBLs that share the same specificity as the sensitizing antigen (e.g., DPw1) and add to the culture system at 2-5 times the number of reagent cells. If the PLT reagents are expanded for a second cycle (one week), it may be advisable to use a different feeder cell donor(s) who shares the sensitizing specificities. In this manner, the expansion of populations primed against weaker, irrelevant specificities should be minimized.
   b. LCLs: LCLs (irradiated 10,000 rads) of the specific stimulator cell or other LCLs should be cultured at least one week prior to use as a feeder cell to ensure proper antigen expression and sterility. It is advisable to culture the LCLs without antibiotics for a period of time prior to addition in culture.
   c. Autologous filler cells: Autologous feeder cells (PBLs) may be used as filler cells in conjunction with antigen-specific PBL or LCL feeder cells at a ratio of 4:1 (auto:specific). Personal communication with several investigators suggests the ^{3}H-Tdr uptake (as the measure of reactivity) may be maintained longer by not over-stimulating the clones.
2. Select Culture Volumes
   The following chart provides guidelines for flask size and culture volumes to be used depending upon the number of PLT reagent cells available.

# PLT Cells x 10^6	# Feeder Cells x 10^6	Flask Size	Max Volume
2	4-10	25 cm^2	20 ml
20	40-100	75 cm^2	100 ml
>20	2-5 x PLT#	490 cm^2	500 ml

3. Add PLT reagent cells and the appropriate number and type of irradiated feeder cells.
4. Dilute reagent cells to 0.3-0.5 x 10^6 cells/ml with filtered culture medium without rIL-2.
5. Incubate at 37° C in a 5% CO_2 humidified environment.
6. When reagent cell concentration exceeds 1 x 10^6 cells/ml, add sufficient medium to adjust the concentration to 0.4 x 10^6/ml.
7. On the third day, add rIL-2 at the appropriate concentration so that the optimal concentration per culture volume is obtained.
8. Continue to adjust cell concentration to 0.4 x 10^6/ml using filtered rIL-2 containing medium. Culture cells for one week.
9. The reagent cells may be expanded by adding the feeder cells weekly and keeping the cells diluted to the appropriate concentration. However, ^{3}H-Tdr incorporation may or may not be maintained.

Variations

1. If lymphoblastoid cell lines (LCLs) are used as secondary stimulator cells, adjust the concentration to 0.1-0.25 x 10^6 LCL/ml. The optimal concentrations vary slightly with each primed reagent and each LCL. Since LCLs are grown in fetal calf serum, be certain the cells are washed a minimum of three times before being added to the culture system. The LCL must be irradiated at a higher dose (10,000 rads).
2. U vs. V bottom plates. The PLT assay system has been described using either U or V bottom plates. For the responder cell concentrations of 10,000 or 5,000 cells/well, either plate works well. The V bottom plates may be slightly better for the lower cell concentrations. The investigator should test which plate works best for his/her test purposes.
3. Label Time. Eight-hr label times with thymidine (2 mCi per well of thymidine at 20 Ci/mM). In addition, 18-hr label times using thymidine at 2 mCi per well of either the 6.5 Ci/mM or 20 Ci/mM concentration have also been described. Again, these variations should be tested by the individual investigator.

Quality Control

Positive Controls

Cells of the original stimulator used to generate the primed reagent should be used as an appropriate secondary stimulatory control for the responding reactivity of the reagent. Alternatively, cells positive for the sensitizing determinants may be substituted. It is advisable to use at least three control cells (for example, for HLA-DP) in case a given positive control cell does not stimulate well.

Negative Controls

1. Medium controls: The responder cells must be tested with the culture medium to determine the levels of background reactivity.

2. Negative control cells: Cells of the original responder used to generate the primed reagent should be used as an appropriate negative secondary stimulatory control. In addition, the investigator should determine an appropriate number of cells negative for the sensitizing determinant to be used to determine the range for the negative response values.

Controls for the varying stimulatory capabilities of the secondary stimulator cells. Three alternative methods have been used to determine the stimulatory capabilities of the stimulator cells: Pool primed PLT, PHA-PLT, and MLC assay. Each provides comparable results. The pool primed PLT procedure is outlined below; however, the choice of the control reagents is at the discretion of the investigator.

Quality Control Procedure: Pool Primed PLT

1. Draw 60 ml sterile heparinized blood from 2 normal cell donors to serve as responders.
2. For the stimulator pool, draw 40 ml sterile heparinized blood from 3 normal cell donors who are disparate with the responders and each other for as many HLA class II specificities as possible. Optimal concentration is 8-10 x 10^6 pooled cells/flask.
3. Isolate mononuclear cells by density gradient centrifugation and resuspend cells in filtered 10% iPHS/RPMI.
4. Irradiate stimulator cells at 3000 rads, then pool the 3 stimulator cells.
5. For each flask add 8-10 x 10^6 pooled stimulator cells in a final volume of 15 ml/flask.
6. Add 2.0 ml filtered 10% iPHS/RPMI to both flasks on days 2, 4, 6, and 8.
7. On day 10, harvest the primed cells. Combine the 2 primed cell populations and rate-freeze at a concentration of 3.5 x 10^6 cells/vial.
8. Pool primed PLT reagents are thawed as usual on the day of the assay and added to the assay at a concentration of 10,000-20,000 cells per well. The pool primed reagent is also cultured in the absence of any stimulator cells to determine the background proliferation of the reagent.
9. Results are analyzed to identify unacceptable low stimulation values as determined by the investigator. Results with stimulator cells eliciting a low restimulation value should be excluded from the interpretation.

Troubleshooting

Serum

One of the most common sources of technical variation which occurs in any cellular assay is a poor serum source. Each individual lot of a serum source, or preferably each individual serum unit comprising the lot, should be screened for growth support capabilities and possible anti-HLA antibodies. The screen should include a control response to a pool of allogeneic cells to measure maximum response, and an autologous control to ensure low backgrounds. If sporadic high backgrounds are observed, an endotoxin test may be advisable.

Results and Interpretation

Results can be expressed in three ways: raw counts per minute (cpm); % relative response (RR); and % reference response. The data may be reduced to allow for easier interpretation and comparability from one test to another.

1. **Relative response** (RR) is calculated as a percentage of the secondary response by the original priming cell after correction for the autologous control as follows:

$$\text{relative response (RR)} = \frac{\text{test cell (cpm)}–\text{autologous cell (cpm)}}{\text{reference cell (cpm)}–\text{autologous cell (cpm)}} \times 100$$

2. **Reference response** is calculated as a percentage of the 75th percentile restimulation value. The % reference responses are plotted for each concentration to provide a further visual analysis. Ideally, a bimodal distribution will occur with all cells which share PLT stimulatory determinants with the original stimulating cell clustering around 100%, and those cells not sharing determinants demonstrating very low restimulation values. The positive versus negative restimulation values are determined by a cluster analysis program.[6]

Perhaps one of the most feasible uses of the PLT in the small cellular testing laboratory is to investigate anomalous MLC reactivity in lieu of HLA-Dw typing with HTCs or HLA-DR and HLA-DP typing by DNA-based methodologies. A well-characterized panel of cells is essential for this type of analysis.

Table 1A illustrates the MLC reactivity observed with cells for two siblings with identical HLA-A, B, C, DR and Dw typing. Although the sibling's cells did not respond significantly to stimulation by the patient's cells (1% RR), cells from the patient responded weakly (9-16% RR) to stimulation by cells from the sibling.

A PLT reagent was generated using the patient's cells as the responding cells and the sibling's cells as the stimulator cells. This reagent was tested with cells from the family as well as 15 unrelated panel cells (Table 1B). Significant restimulation, as determined by cluster analysis,[6] correlated with the presence of the DP2 specificity in the sibling but not the patient as well as in 3 unrelated cell donors. If a laboratory does not have a Dw, DP typed panel of cells, this type of analysis can still be performed. A DP disparity can be postulated based on family segregation analysis, lack of correlation with a DR specificity, as well as inhibition of reactivity in the presence of anti-DP monoclonal antibody.[28,40]

Table 1. PLT as an Adjunct Test for MLC Assays

A. Slight MLC reactivity between cells from HLA-A, B, C, DR, Dw identical siblings. Patient and sibling are HLA-A2, 3; B13, 35; DR/Dw7, 8.

		Autol[3] - Control$_x$	**Pool[4] + Control$_x$**	**Patient$_x$**	**Sibling$_x$**
Patient 50,000 cells/well	cpm[1]	866	58,346	1,336	6,046
	RR[2]	0	100%	0%	9%
Patient 25,000 cells/well	cpm	413	41,742	436	7,218
	RR	0	100%	0%	16%
Sibling 50,000 cells/well	cpm	532	45,502	1,294	1,094
	RR	0	100%	1%	1%

B. *PLT Reactivity*: Patient's cells primed against sibling cells

	Dw	**DP**	**cpm**	**RR**
Stimulator cells x[5]				
Patient	7,8	NT	2,368	0
Sibling	7,8	NT	26,386	100
Father	8	NT	5,972	13
Mother	7	NT	44,316	156
DK	2,3	2,4	34,462	125
NR	9,14	2,6	26,916	92
HH	1,3	2,4	23,632	75

Negative restimulation cpm 4,956 – 19,044
RR 10-67
N = 12

[1]cpm = results expressed as counts per minute tritiated thymidine incorporation
[2]RR = relative response
[3]Autol = stimulation by autologous x-irradiated cell
[4]Pool = stimulation by a pool of 3 unrelated control cells
[5]x = x-irradiated cells

Table 2 illustrates another investigation of anomalous MLC reactivity. Family HLA testing revealed the patient had inherited a recombinant haplotype such that the patient's and sibling's cells were HLA-D region identical and disparate for HLA-B (Table 2A). The MLC results indicated a significant response by the sibling's cells to stimulation by the patient's cells. A PLT reagent was generated using the sibling's cells as responder cells and the patient's cells as stimulator cells. Significant PLT reactivity was correlated with the HLA-B62 specificity. Subsequently, T cell clones were identified which demonstrated PLT and/or cell-mediated lympholysis (CML) reactivity specific for HLA-B62. Class I – directed reactivity is not often observed in the MLC assay.

Further Applications

PLT has been used to detect donor antigen-specific reactivity of bronchoalveolar lavage (BAL) lymphocytes associated with acute lung rejection and obliterative bronchiolitis (OB), as well as cells infiltrating transplanted renal allografts,[16] liver and cardiac allografts,[13,42] and skin biopsies obtained at the site of a graft versus host disease (GVHD) lesion.[24] Propagation of T lymphocytes from renal, cardiac, and hepatic allografts demonstrates a strong correlation between long-term T cell growth and the clinical presence of acute cellular rejection. PLT has been used to investigate the specificity of these graft infiltrating cells.[19] Our previous studies[23] demonstrated a predominant $CD8^+$ cell population-mediated class I donor antigen-specific reactivity correlating with OB in 3/3 recipients tested, and a predominant $CD4^+$ cell population-mediated class II donor antigen-specific reactivity correlating with acute rejection episodes in 13/15 recipients tested. These studies are of importance not only in monitoring recipients, but also in investigating the immunological basis of pulmonary disease. Take together, these results suggest that distinct immunopathogenetic events may be occurring during acute lung rejection and OB. Thus, this technique has been used to demonstrate functional characteristics of graft infiltrating cells, and to provide information regarding the activation state of the T cell infiltrate.

In conclusion, the PLT assay described in this chapter has been used historically for the investigation of T cell recognized epitopes. This technique remains useful for assessing T cell recognized epitopes and will undoubtedly provide valuable information in evaluating the immune status of transplant recipients.

Table 2. PLT as an adjunct to MLC Assays

A. MLC reactivity between cells from HLA-D region identical siblings
Patient JB = HLA-A32, 1; B37, 62; DR/Dw6, DR/Dw7
Sibling KS = HLA-A32, 1; B37, 17; DR/Dw6, DR/Dw7

Responder	Testing #		Autol[3] - Controlx	Pool[4] + Control	Patient$_x$[5]	Sibling$_x$
Patient	1	cpm[1] RR[2]	1,384 0	62,363 100%	– –	1,213 -3%
	2	cpm RR	3,478 0	57,135 100%	– –	3,150 -6%
Sibling	1	cpm RR	594 0	53,266 100%	9,306 16%	– –
	2	cpm RR	671 0	63,665 100%	13,634 20%	– –

B. PLT Reactivity: Sibling's cells primed against patient's cells

	HLA-B	cpm	RR
Sibling	17	482	0
Patient	62	23,022	100
GP	62	40,500	173
NE	62	53,158	237
CS	62	42,598	187
KB	63	554	2
FB	15 neg	6,542	23
BV	15 neg	846	2

[1]cpm = results expressed as counts per minute tritiated thymidine incorporation
[2]RR = Relative Response
[3]Autol = stimulation by autologous x-irradiated cell
[4]Pool = stimulation by a pool of 3 unrelated control cells
[5]$_x$ = x-irradiated cells

References

1. Anderson LC, Hayry P, Specific priming of mouse thymus dependent lymphocytes to allogenic cells in vitro. Eur J Immunol 3:595, 1973.
2. Bach FH, Bradley BA, Yunis EJ, Response of primed LD typing cells to homozygous typing cells. Scand J Immunol 6:477, 1977.
3. Bach FH, Reinsmoen NL, Cloned cellular reagents to define antigens encoded between HLA-DR and glyoxalase. Hum Immunol 5:133, 1982.
4. Bach FH, Reinsmoen NL, Segall M, Definition of HLA antigens with cellular reactants. Transplant Proc 15:102, 1983.
5. Bach FH, Sondel PM, Sheehy MJ, Wank R, Alter BJ, Bach ML, The complexity of the HL-A LD system: a PLT analysis. In: *Histocompatibility Testing 1975*; F Kissmeyer-Nielsen, ed.; Munksgaard, Copenhagen, p. 576, 1975.
6. Carroll PG, DeWolf WC, Mehta CR, Rohan JE, Yunis EJ, Centroid cluster analysis of the primed lymphocyte test. Transplant Proc 11:1809, 1979.
7. DeWolf WE, Carroll PG, Mehta CK, Martin SL, Yunis EJ, The genetics of PLT response. II. HLA-DRw is a major PLT-stimulating determinant. J Immunol 123:37, 1979.
8. Duquesnoy RJ, Zeevi A, Marrari M, Halim K, Immunogenetic analysis of the HLA-D region: Serological and cellular detection of the MB system. Clin Immunol Immunopathol 23:254, 1982.
9. Eckels DD, Johnson AH, Hartzman RJ, Dacek D, Clonal analysis of HLA-DPw1 (SB1) associated allodeterminants. I. Recognition of novel epitopes and evidence for quantitative variation in class II antigen expression. Hum Immunol 15:234, 1985.
10. Flomenberg N, Naito K, Duffy E, Knowles RW, Evans RL, Dupont B, Allocytotoxic T-cell clones: Both leu 2+3- and 2-3+ T cells recognize class I histocompatibility antigens. Eur J Immunol 13(11):905, 1983.
11. Fradelizi D, Dausset J, Mixed lymphocyte reactivity of human lymphocyte primed in vitro. I. Secondary response to allogeneic lymphocytes. Eur J Immunol 5:295, 1975.
12. Fradelizi D, Nunez-Roldan A, Sasportes M, Human Ia-like Dw lymphocyte antigens stimulating activity in primary mixed lymphocyte reaction. Eur J Immunol 8:88, 1978.
13. Fung JJ, Zeevi A, Starzl TE, Demetris J, Iwatsuki S, Duquesnoy RJ, Functional characterization of infiltrating T lymphocytes in human hepatic allografts. Hum Immunol 16: 182, 1986.
14. Hartzmann RJ, Pappas F, Romano PJ, Johnson AH, Ward FE, Amos DB, Disassociation of HLA-D and HLA-DR using primed LD typing. Transplant Proc 10:809, 1978.

15. Mawas C, Charmot D, Sasportes M, Secondary responses of in vitro primed human lymphocytes to allogenic cells. I. Role of 6 HL-A antigens and mixed lymphocytes reaction stimulating determinants in secondary in vitro proliferative responses. Immunogenetics 2:449, 1975.
16. Miceli C, Barry TS, Finn OJ: Human allograft derived T-cell lines, donor class I- and class II-directed cytotoxicity and repertoire stability in sequential biopsies. Hum Immunol 22:185, 1988.
17. Morling N, Jakobsen BK, Platz P, Ryder LP, Svjgaard A, Thomsen M, A "new" primed lymphocyte testing (PLT) defined DP-antigen associated with a private HLA-DR antigen. Tissue Antigens 16:95, 1980.
18. Pawlec G, Shaw S, Schneider M, Blaurech M, Frauer M, Brackerts D, Wernet P, Population studies of the HLA-linked SB antigen and their relative importance to primary MLC-typing analysis of HLA-D homozygous typing cells and normal heterozygous populations. Hum Immunol 5:215, 1982.
19. Rabinowich H, Zeevi A, Paradis IL, Yousen SA, Dauber JH, Kormos R, Hardesty RL, Griffith BP, Duqesnoy RV, Proliferative responses of bronchoalveolar lavage lymphocytes from heart-lung transplant patients. Transplantation 49:115, 1990
20. Reinsmoen NL, Anichini A, Bach FH, Clonal analysis of T lymphocyte response to an isolated class I disparity. Hum Immunol 8:195, 1983.
21. Reinsmoen NL, Bach FH, HLA-D region complexity associated with HLA-DR, Dw and SB phenotypes. Transplant Proc 15:76, 1983.
22. Reinsmoen NL, Bach FH, Clonal analysis of HLA-DR and -DQ associated determinants – their contributions to Dw specificities. Hum Immunol 16:239, 1986.
23. Reinsmoen NL, Bolman RM, Savik K, Butters K, Hertz M, Differentiation of class I- and Class II directed donor-specific alloreactivity in bronchoalveolar lavage lymphocytes from lung transplant recipients. Transplantation 53:181, 1992.
24. Reinsmoen NL, Kersey J, Bach FH, Detection of HLA restricted anti-minor histocompatibility antigen(s) reactive cells from skin GVHD lesions. Hum Immunol 11:249, 1984a.
25. Reinsmoen NL, Kersey J, Yunis EJ, Antigens associated with acute leukemia detected in the primed lymphocyte test. J Nat Cancer Inst 60(#3):537, 1978.
26. Reinsmoen NL, Layrisse Z, Beutel H, Bach FH, A study of HLA-DR2 associated HLA-Dw/LD specificities. Hum Immunol 11:105, 1984.
27. Reinsmoen NL, Noreen HJ, Sasazuki T, Segal M, Bach FH, Roles of HLA-DR and HLA-D antigens in haplo-primed LD typing reagents. Proceedings of the 13th International Leukocyte Culture Conference. In: *The Molecular Basis of Immune Cell Function*; Elsevier, Amsterdam, p. 529, 1979.
28. Royston I, Omary MB, Trobridge IS, Monoclonal antibodies to a human T-cell antigen and Ia-like antigen in the characterization of lymphoid leukemia. Transplant Proc 13:761, 1981.
29. Sasportes M, Nunez-Roldan A, Fradelizi D, Analysis of products involved in primary and secondary allogenic proliferation in man. III. Further evidence for products different from Ia-like DRw antigens, activating secondary allogenic proliferation in man. Immunogenetics 6:55, 1978.
30. Shaw S, Duquesnoy R, Smith P, Population studies of the HLA-linked SB antigens. Immunogenetics 14:153, 1981.
31. Shaw S, Johnson, AH, Shearer GM, Evidence for a new segregant series of B cell antigens that are encoded in the HLA-D region and that stimulate secondary allogeneic proliferative and cytotoxic responses. J Exp Med 152:565, 1980a.
32. Shaw S, Pollak MS, Payne SM, Johnson AH, HLA-linked B-cell alloantigens of a new segregant series: Population and family studies of the SB antigens. Hum Immunol 1:177, 1980b.
33. Sheehy MJ, Sondel PM, Bach ML, Wank R, Bach FH, HL-A LD (lymphocyte defined) typing: A rapid assay with primed lymphocytes. Science 188:1308, 1975.
34. Sheehy MJ, Bach FH, Primed LD typing (PLT) – Technical considerations. Tissue Antigens 8:157, 1976.
35. Suciu-Foca N, Complete typing of the HLA region in families. IV. The genetics of HLA-D as seen by HTC and PLT methods. Transplant Proc 9:1751, 1977.
36. Suciu-Foca N, Susnno E, McKiernan P, Rohowsky C, Weiner J, Rubinstein P, DRw determinants on human T cells primed against allogeneic lymphocytes. Transplant Proc 10:845, 1978.
37. Thompson JS, Easter CH, Balschke JW, Use of primed lymphocyte (PLT) in unrelated individuals to identify 11 antigenic clusters. Transplant Proc 9(4): 1759, 1977.
38. Wank R, Schendel DJ, Blanco ME, Dupont B, Secondary MLC responses of primed lymphocytes after selective sensitization to non-HLA-D determinants. Scand J Immunol 9:499, 1979.
39. Wank R, Schendel DJ, Hansen JA, Dupont B, The lymphocyte restimulation system: Evaluation by intra-HLA-D group priming. Immunogenetics 6:107, 1978.
40. Watson AJ, Demars R, Trobridge IS, Bach FH, Detection of a novel human class II HLA antigen. Nature (Lond.) 304:358, 1983.
41. Zeevi A, Duquesnoy RJ, PLT specificity of alloreactive lymphocyte clones for HLA-B locus determinants. Proc Natl Acad Sci USA 80:1440, 1983.
42. Zeevi A, Fung J, Zerbe TR, Kaufman C, Rabin BS, Griffith BP, Hardesty RL, Duquesnoy RJ, Allospecificty of activated T cells grown from entomyocardial biopsies from heart transplant patients. Transplantation 41:620, 1986.
43. Zeevi A, Scheffel C, Annen K, Bass G, Marrari M, Duquesnoy RJ, Association of PLT specificity of alloreactive lymphocyte clones with HLA-DR, MB and MT determinants. Immunogenetics 16:209, 1982.

In Vitro Measurements of Cell-Mediated Cytotoxicity: Cytotoxic Effector Cells

Sandra W. Helman and Malak Y. Kotb

Purpose

The immune response to many viruses and other intracellular pathogens, as well as the response to tumors and transplanted tissue, involves the elicitation and activity of cytotoxic effector cells, which are responsible for the destruction of foreign, malignant, or infected cells and the accompanying immunopathology.

The cells that mediate cytotoxicity in the human are varied in their origin and the mechanism of their activation.[1-3] They may be T cells, NK (natural killer) cells, monocytes/macrophages, or granulocytes. Cytotoxicity may be specifically elicited due to recognition of peptides in association with Class I Major Histocompatibility Complex (MHC) molecules by cytotoxic $CD8^+$ T cells and Class II molecules by cytotoxic $CD4^+$ T cells (CTLs), or by recognition of Fc receptors on the surface of specific antibody coated target cells by Fc receptor-bearing K (ADCC killer) cells. In contrast to CTLs, K cells may be of T cell, NK cell, or monocyte/macrophage origin. Other cytotoxic cells may occur without prior stimulation or priming.

NK cells, as the name implies, do not require prior sensitization to recognize and kill their targets. Killing by NK cells occurs in an MHC-unrestricted manner. NK cells are responsive to a number of cytokines, such as IL-2, which can increase their cytotoxic activity. Activation by high doses of IL-2 can also induce MHC-unrestricted killing by lymphokine activated killer cells (LAK cells), and by certain subsets of T cells exhibiting NK-like activity. Monocytes and macrophages may also be nonspecifically activated by cytokines or other stimuli to kill a variety of target cells.

The specific responses will be shaped by the MHC antigens of the responde.[4] Cytotoxic T lymphocytes (CTL) are generated following stimulation of precursor T cells by specific antigen presented by MHC class I molecules. They kill target cells expressing the sensitizing antigen in an MHC restricted manner. Circulating CTL precursors are not fully differentiated when they exit the thymus. Differentiation requires exposure to a sensitizing antigen. Normally, the presence of functional CTLs specific for an allograft is very difficult to detect in the blood of a potential recipient. Prior exposure to allograft antigens either due to the blood transfusions or previous transplants can significantly increase the number of allograft-specific CTLs. In vitro exposure to alloantigens for 7-10 days, as in mixed leukocyte reactions (MLR) may result in expansion and differentiation of donor-specific CTLs, thereby greatly facilitating their detection. During the in vitro incubation period helper $CD4^+$ cells respond directly to allogeneic MHC class II molecules, become activated, secrete cytokines, and proliferate. The binding of CTL precursors to alloantigen presented by MHC class I molecules triggers signals that in concert with cytokine-induced signals results in the differentiation of CTL. The CTL are now ready to perform their effector function, which is to kill the target cells expressing the sensitizing alloantigen.

The ability of each individual to respond to viral infections, or to mount a cellular reaction to a tumor or to transplanted tissue will depend on the numbers and functionality of all of these cells.

Natural Killer (NK) Cell Assay

Purpose

The purpose of the Natural Killer (NK) Cell Assay is to assess the activity of spontaneous killer cells present in the peripheral blood. NK cells are an important part of the host defense against viruses and tumors. Abnormalities in NK function can result from various causes, including primary or secondary immunodeficiency, the presence of a large tumor mass, stress, and autoimmune disease1. A variety of cytokines and other factors can increase NK activity, and assessment of NK responses before and after treatment with these factors may be a measure of their clinical efficacy.[3]

Specimen

1. Collect 20 ml of heparinized whole blood from the patient and store at room temperature. Isolate within 24 hrs after collection. Isolated mononuclear cells may be frozen and stored in liquid nitrogen for later testing if required. This is not recommended because of potential losses in activity.
2. Run a control sample at the same time as the patient sample (see Interpretation section for appropriate controls).

Unacceptable Specimen

Blood must be received in the laboratory no more than 18 hr after collection. Whole blood that has been refrigerated or exposed to heat is not acceptable.

Supplies and Instrumentation

1. **Supplies**
   a. 96 well round or V bottom tissue culture plates
   b. 15 ml sterile plastic centrifuge tubes
   c. 50 ml sterile plastic centrifuge tubes
2. **Instrumentation**
   a. CO_2 incubator
   b. Gamma counter or beta counter
   c. Centrifuge with microtiter plate carriers

Reagents

1. Tissue culture media
   a. Fetal bovine serum (FBS): Must be heat-inactivated (HI) prior to use. Heat inactivate by incubation in a 56° C waterbath for 30 min. Aliquot and store frozen until needed.
   b. 30% N-[2-Hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid] (HEPES) buffer: Weigh out 30 g of HEPES and dissolve in 100 ml distilled water. Sterilize by filtration.
   c. Complete RPMI 1640:

RPMI 1640	100 ml
FBS-HI	5 ml
Glutamine 200mM	5 ml
Gentamicin	0.25 ml
30% HEPES	5 ml

   d. Complete McCoy's 5A:

McCoy's 5A	100 ml
FBS-HI	5 ml
Glutamine 200mM	5 ml
Gentamicin	0.25 ml
30% HEPES	5 ml

2. Other reagents
   a. 5% Triton X 100 (TX100): Add 5 ml Triton X detergent to 95 ml distilled water.
   b. ^{51}Chromium (^{51}Cr): 1 mCi/ml. Commercially available. ^{51}Cr has a half-life of 27.7 days. Each lot of ^{51}Cr should be accompanied by a calibration date. It is necessary to determine the remaining activity on the day used (see Calculations).
   c. Target Cells: The K562 cell line is the standard target cell for the NK cell assay. It may be obtained from the American Type Culture Collection (ATCC, Rockville, MD). Maintain cells in complete RPMI 1640 by passaging twice per week by resuspending at a concentration of 1 x 10^5 cells/ml. A supply of vials of frozen cells from a low passage number should be stored in liquid nitrogen.
   d. Ficoll-Hypaque (FH) or Lymphocyte separation medium (LSM): Density 1.077-1.080.

Procedures

1. **Preparation and Labelling of Target Cells**
   a. K562 cells are used 72 hrs after passaging of cultures under the conditions described. Cell viability should be checked prior to labelling and should be >80%.
   b. Remove an aliquot of cells from the culture containing 4 x 10^6 cells/ml and wash 1X with McCoys medium.
   c. Discard the supernatant and resuspend in 0.6 ml of McCoys medium and add 150 µCi ^{51}Cr (adjusted for decay).
   d. Incubate cells with ^{51}Cr in a 37° C CO_2 incubator for 1.5 hr, agitating the cells gently every 30 min.
   e. After incubation, underlay with 4 ml of FBS-HI and centrifuge at 800 RPM in a refrigerated centrifuge (4-8° C). Collect the cell pellet.
   f. Wash cells twice with cool complete McCoys medium in the cold. Resuspend after final wash in complete RPMI.
   g. Adjust labelled targets to a concentration of 5 x 10^4 cells/ml and set aside three 100 µl samples of cells. Keep remainder at 4° C until plated.
   h. Take the three samples of cells set aside in **Step g** and count in a gamma counter to determine if cells are adequately labelled. Counts should be between 500 and 10,000 CPM. See troubleshooting if labelling is inadequate.
   Note: RADIATION SAFETY RULES MUST BE FOLLOWED WHEN WORKING WITH ^{51}Cr (see Radiation Safety chapter).
2. **Isolation of Effector Cells**
   a. Dilute heparinized blood 1:2 with McCoys medium and underlay with LSM.
   b. Centrifuge at 400 x g for 15 min. Remove cell layer at the plasma-LSM interface and wash with complete McCoys medium.

c. Resuspend cells in complete RPMI adjusting to 3 x 10^6 effector cells/ml (at least 1.0 ml of cells is needed for the assay). Make 3 serial twofold dilutions of the cells. This will provide cells at concentrations appropriate for effector/target cell (E:T) ratios of 60:1, 30:1, 15:1, and 7.5:1.

3. **Preparation and Harvest of Cultures**
   a. All cells are plated in 96 well round or V bottom tissue culture plates.
   b. When ready to plate, add 100 µl of target cells to all wells.
   c. Plate effector cells with target cells by adding 100 µl of each dilution to triplicate wells. Test cells from appropriate controls in each run.
   d. As additional controls, plate triplicate wells containing only medium and target cells to determine the spontaneous ^{51}Cr release. Control wells containing target cells and 100 µl TX100 are used to determine the maximum release.
   e. Centrifuge plates for 5 min at 500 RPM and incubate for 4 hr at 37° C in a CO_2 incubator. If cells become dislodged after incubation, centrifuge again prior to harvest.
   f. Collect 100 µl of supernatant from each well and place in a counting vial. Count on a gamma counter using the ^{51}Cr window. Alternatively, samples may be added to scintillation fluid and counted in a beta scintillation counter.

Troubleshooting

1. **Use of Other Target Cells**
   Target cells other than K562 may be appropriate NK targets. However, if other targets are used, optimal conditions of labelling, E:T ratios, cell culture and other aspects of this procedure will need to be determined.
2. **Poor Labelling**
   K562 cells generally label within the parameters indicated above. If labelling is inadequate it may be due to several possibilities.
   a. ^{51}Cr decayed beyond usefulness. Half-life is 27.7 days. Adjustments must be made for decay when labelling (see Table 1). ^{51}Cr that is >30 days beyond assay date may not provide adequate labelling.
   b. Cells may not be at optimal point in growth cycle. Check optimum time for labelling after culture division in your laboratory (cells label best in log phase). Times from 24-72 hrs may be appropriate.
   c. If cells do not label well, and above suggestions are not appropriate, try adding label directly to the dry cell pellet. This may result in a higher labelling efficiency.

Table 1: Decay table for ^{51}chromium

	Days After Calibration									
	0	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
0	1.00	0.975	0.951	0.928	0.905	0.882	0.861	0.839	0.819	0.789
10	0.779	0.760	0.741	0.722	0.705	0.687	0.670	0.654	0.638	0.622
20	0.606	0.591	0.577	0.563	0.549	0.535	0.522	0.509	0.496	0.484
30	0.472	0.461	0.449	0.438	0.427	0.417	0.406	0.396	0.387	0.377

To use the decay tables, find the number of days after the calibration date by using the top and left hand columns, then find the corresponding decay factor.

3. **High Spontaneous ^{51}Cr Release**
   a. May be due to low cell viability. Procedures that increase cell viability by removal of dead cells may not be useful because remaining cells may be too old and fragile. Solution: Repeat with fresh targets with >90% viability. Make sure cells are in log phase of cell growth.
   b. May be due to unbound ^{51}Cr in the cell preparation. Solution: More extensive washes of the cell preparation may be necessary.
4. **Little or No ^{51}Cr Release From All Control and Patient Samples**
   Use of human serum instead of fetal calf serum may cause a poor cytotoxic response. Human IgG has been shown to inhibit NK activity. Solution: Use FBS in all NK assays.
5. **Need to Hold Sample for Testing at a Later Time**
   If possible, all samples should be tested on the same day drawn. Samples that cannot be tested on the same day drawn may be held for testing within 24 hrs, if maintained under the following conditions.
   a. Samples can be tested up to 18 hrs after separation of mononuclear cells. Ideally, if all samples are held for next day testing, control values should be determined on similar samples.
   b. If a sample is received late in the day or if testing cannot be performed within 18 hrs on a sample, the sample can be frozen for testing at a later date. However, freezing of samples may have unpredictable effects on the ability of cells to kill targets[2]. Therefore, it is important to freeze a fresh control with the patient sample to control as much as possible for the effects of freezing.

Interpretation

1. **Controls**
   Controls used for NK analysis should include one or more of the following samples:
   a. A fresh normal control, preferably from a group of previously tested volunteers. NK activity of PBMC from normal individuals remains relatively stable over time.
   b. One or more frozen samples from individuals with known high, low, or intermediate NK activity.
   c. Using a combination of fresh and frozen cells as controls is optimum. Under these circumstances, the assay is invalidated only if both fresh and frozen controls fail. This will allow for problems with recovering cells from the freezer on a given day or for biological variation of a fresh sample due to unknown variables.
   d. Patient and control samples should always be drawn at approximately the same time every day (particularly with serial monitoring) since diurnal variation in NK activity may occur.
2. **K562 Labelling**
   a. For an assay to be valid, spontaneous release from K562 cells must be <20% of maximal release; <10% is optimal.
   b. K562 cells should label with 500 to 10,000 CPM/5 x 10^3 cells for proper interpretation of this assay. If labelling is less than 1000 CPM, spontaneous release approaching 20% may make proper interpretation more difficult.
3. **Responses in Patient**
   Many conditions can cause depressed NK activity. Treatment of patients with intravenous immunoglobulin can severely depress NK function. Many viral infections can also affect NK function. HIV patients may show a significant depression in NK activity without a decrease in NK cell numbers.

   NK activity appears to play a role in resistance to malignancy, both as a protective factor in the development of certain malignant diseases, and as a prognostic indicator of the likelihood of metastasis or relapse.

   It may be useful for flow cytometry testing to be done at the same time to assess the numbers of natural killer cells present when this test is performed. This may help in the interpretation of the test by differentiating between a low response due to few natural killer cells and that due to poorly functional or nonfunctional cells.

Cell Mediated Lympholysis (CML) Assay

Purpose

Cell mediated acute allograft rejection depends on the immunogenicity of the graft and the presence of recipient alloreactive T cells capable of damaging the graft. The Cell Mediated Lympholysis (CML) Assay allows one to determine the presence of cytotoxic T cells (CTL) which can be detrimental to the host and to graft outcome. In addition, this assay can be used to determine MHC compatibility between donor and recipient. Disparities at the MHC loci have been shown to cause acute allograft rejection, and in the case of bone marrow transplantation, can cause graft versus host disease (GVHD) with consequent fatality. Measurement of effector function is done by determining the extent of target cell killing. The most widely used assay for CTL activity is the ^{51}Cr-release assay. Target cells are labelled with ^{51}Cr and then incubated with CTL; the release of ^{51}Cr into the cell-free medium correlates with the degree of target cell lysis.

Specimen

Samples should be obtained from the responder and the allogenic stimulator. Responder cells may be fresh or frozen mononuclear cells from 20 ml of sterile, heparinized (500 U of preservative-free heparin) peripheral blood. Allogenic stimulator cells may be fresh or frozen mononuclear cells from 20 ml of heparinized peripheral blood, or from lymph nodes or spleen. A pool of stimulator cells used as control usually consists of frozen mononuclear cells from heparinized peripheral blood. Frozen mononuclear cells should be isolated prior to freezing and stored in liquid nitrogen.

The cell viability as judged by trypan blue dye exclusion should be ≥ 90%.

Unacceptable Specimen

Highly hemolyzed specimens or specimens with viability below 70% are usually unacceptable. Viability between 70-90% may be acceptable if additional cells are unavailable. Recollection of these specimens is preferable, when possible.

Reagents

1. Serum supplements:
   a. Fetal bovine serum (FBS) is commercially available and must be heat-inactivated (HI) prior to use. Heat inactivate by incubation in a 56° C waterbath for 30 min. Aliquot and store frozen until needed.
   b. Human AB serum (HS) is commercially available. Serum should be from a pool from untransfused males.
2. Media and media supplements
   a. 30% HEPES buffer: Weigh out 30 g of HEPES and dissolve in 100 ml distilled water. Sterilize by filtration.

b. Complete RPMI 1640:

RPMI 1640	100 ml
FBS-HI or	10 ml or
HS	15 ml
Glutamine 200mM	5 ml
Penicillin/streptomycin	5 ml
30% HEPES	12.5 ml

c. Hanks Balanced Salts Solution (HBSS): Commercially available. Adjust pH to 7.0.

2. Other reagents
   a. Tritiated thymidine (^{3}H-Tdr). Commercially available.
   b. 5% Triton X 100 (TX100): Add 5 ml Triton X detergent to 95 ml distilled water.
   c. ^{51}Chromium (^{51}Cr): 3 mCi/ml. ^{51}Cr has a half-life of 27.7 days. Each lot of ^{51}Cr should be accompanied by a calibration date. It is necessary to determine the activity on the day used (see Calculations Section).
   d. Allogeneic stimulator cell pool: The pool usually consists of a mixture of 5-6 unrelated cells which are DR-disparate. They are prepared in bulk and frozen in liquid nitrogen in 1 ml aliquots of $5 \times 10^6 - 1 \times 10^7$ cells per vial. A supply of vials of frozen cells should be stored in liquid nitrogen.
   On day needed, thaw the vials for each experiment by rapidly shaking the vials in a 40° C water bath until the contents of the vial have just thawed. Immediately transfer the cells to a 15 ml sterile tube containing prewarmed complete RPMI medium. Gently mix the cells and centrifuge the tube at 250 x g for 8 min. Resuspend the cells in complete RPMI medium and place in a 37° C incubator for 30-60 min. Check cell viability and adjust the concentration to 1×10^6 cells/ml.
   e. Ficoll Hypaque (FH) or Lymphocyte separation medium (LSM): Density 1.077-1.080.
   f. Phytohemagglutinin (PHA): Optimal concentration for blast generation should be determined before use.
   g. Mitomycin C: Store in the dark at 4° C after reconstitution.

Supplies and Instrumentation

1. **Supplies**
   a. 96 well round bottom tissue culture plates
   b. 24 well tissue culture plates (3 ml, 16 mm) or 50 ml tissue culture flasks
   c. 15 ml sterile conical plastic tissue culture tubes
   d. 50 ml sterile plastic centrifuge tubes
2. **Instrumentation**
   a. Gamma counter
   b. Beta scintillation counter
   c. CO_2 incubator
   d. Laminar flow hood

Procedures

1. **Summary of Steps**

DAY 0: Preparation of effector cells

a. Prepare responder cells.
b. Inactivate the stimulator cells with mitomycin C or irradiation (see MLC Chapter).
c. Set up target cell cultures.
d. Co-culture responder and inactivated stimulator cells for 6 days to generate effector CTL.

DAY 2: Preparation of target cells

Add PHA to target cell cultures.

DAY 6: Set up cytotoxic cell assay

a. Harvest target cells and label with ^{51}Cr.
b. Harvest effector cells.
c. Incubate effector and target cells in CTL assay for 5 hrs.
d. Harvest supernatants and count ^{51}Cr release.

2. **Isolation of Responder and Stimulator Cells**
   a. Be sure to perform all steps of this procedure using sterile reagents and with sterile procedure.
   b. Dilute 20 ml of heparinized blood from each stimulator and responder by adding 10 ml HBSS. Mix gently.
   c. Prepare and label 50 ml sterile tubes for each blood specimen. Fill each tube with 20 ml LSM. Tilt the tubes to a 30° angle and gently overlay the LSM with the diluted blood allowing the blood to flow slowly and steadily down the inside of the tube. Alternatively, underlay the blood with LSM.
   d. Centrifuge the tubes for 20 min at 900 x g. It is important to increase the acceleration gradually and to allow the centrifuge to come to a halt without braking.
   e. The peripheral blood mononuclear cells (PBMC) will form a layer at the interface between the lower LSM layer and the upper platelet-rich plasma layer.

f. Carefully recover the cells at the interface and transfer to a sterile 50 ml tube. Add 30-40 ml HBSS, mix gently and centrifuge at 250 x g for 8 min.
g. Remove the supernatant by aspiration and resuspend the pellet in 1 ml HBSS. Pipet up and down gently to dislodge the pellet and resuspend the cells, avoiding clumping. Add 20 ml of HBSS, mix gently and centrifuge again at 250 x g for 8 min. Repeat this procedure once more for a total of three HBSS washes.
h. Resuspend the final pellet in 1 ml complete RPMI. Add an additional 4 ml RPMI medium and mix gently.
i. Determine the number of viable cells using trypan blue dye exclusion and adjust the cell concentration to 10^6 cells/ml in complete RPMI.
j. From the specific allogeneic cell preparation remove an aliquot of 5 x 10^6 cells for treatment as specific allogeneic stimulator cells and 2 x 10^6 cells to be used as specific allogeneic target cells.
k. From the responder cell preparation aliquot 5 x 10^6 cells for treatment as autologous stimulator cells and 1 x 10^6 cells to be cultured with PHA to serve as autologous target cells. The remainder of the cells are ready to use as responder cells (at least 10 ml will be required). Store on ice until needed.

2. **Preparation of Stimulator Cells**
   a. Samples to be treated as stimulator cells are reserved aliquots of 1) specific allogeneic cells, 2) autologous cells, and 3) a pool of allogeneic stimulator cells.
   b. Treat 5 x 10^6 cells from each group of stimulators with mitomycin C to arrest their proliferation.
   c. Incubate the cells in a tube with 25 mg/ml mitomycin C for 30 min at 37° C with occasional shaking. Protect from light.
   d. Wash the cells 5 times with HBSS. For each wash resuspend the pellet in 1 ml HBSS, then add 9 ml HBSS, mix and centrifuge at 250 x g for 8 min. After the final wash resuspend the pellet in complete RPMI and adjust the cell concentration to 1 x 10^6 cells/ml.
   e. An **ALTERNATIVE** method to block stimulator cell proliferation is to irradiate the cells with 2000 rad, followed by a single wash in HBSS.
   f. To determine if either the mitomycin C treatment or the irradiation was successful, culture triplicate wells of 1 x 10^5 inactivated stimulator cells with 1 mg PHA in a total of 200 µl in 96 well microtiter plates. Measure proliferation by ^{3}H-Tdr uptake at 72 hrs.
3. **Generation and Harvesting of Effector CTL**
   a. In each well of 24 well tissue culture plates combine 1 ml of responder cells with 1 ml of mitomycin C-treated stimulator cells (specific allogeneic cell, autologous cells, or allogeneic cell pool). Plate 3 wells for each responder/stimulator pair.
   b. **ALTERNATIVE PROCEDURE.** For BULK cultures, mix 10 ml of responder cells with an equal volume of stimulator cells in 50 ml tissue culture flasks.
   c. Incubate the plates or flasks for 6 days at 37° C in the presence of 5% CO_2 and 95% humidity.
   d. At the end of 6 days, harvest the effector cells by forceful resuspension of the cells. Combine the CO_2 cell suspension from triplicate wells in a 15 ml conical sterile tube. Wash wells once with HBSS and add to tubes.
   e. Centrifuge tubes at 250 x g for 8 min.
   f. Resuspend the pellet in 1 ml of complete RPMI. Count the cells with trypan blue.
   g. Adjust the effector cells to 1 x 10^6 cells/ml, 5 x 10^5/ml, 2.5 x 10^5/ml and 1.25 x 10^5/ml in complete RPMI.
4. **Preparation and Harvest of Target Cells**
   a. On day 0, plate 2 x 10^6 of each target cell (autologous, specific allogeneic, and pooled allogeneic) in a 24 well sterile tissue culture plate and incubate for 2 days in complete RPMI at 37° C in a humidified CO_2 incubator.
   b. After 2 days, add 0.5 µg/ml PHA to the targets and further incubate for an additional 4 days. Normal resting cells do not label well with ^{51}Cr, however, PHA stimulation increases the efficiency of ^{51}Cr labelling.
   c. On day 6, transfer each PHA-stimulated target to a 15 ml sterile conical tube, then wash the original wells with HBSS, and combine the wash with the harvested cells. Fill the tube with complete RPMI.
   d. Centrifuge the tubes at 250 x g for 8 min, then aspirate the supernatant off, leaving 50-100 µl behind. Flick the tube gently to resuspend the pellet.
   e. Add 100 µl of ^{51}Cr to each pellet, followed by 20 µl of HS or 20 µl of FBS and gently mix the cells.
   Note: RADIATION SAFETY RULES MUST BE FOLLOWED WHEN WORKING WITH ^{51}Cr. (See Radiation Safety chapter)
   f. Loosen the cap of the tube and incubate at 37° C in a humidified CO_2 incubator for 1 hr. After 30 min of incubation, flick the tube gently to ensure that the cells are well suspended.
   g. Wash the target cells 3 times by first resuspending the cells in 1 ml complete RPMI, then filling the tubes with medium, mixing the cells and centrifuging the tubes at 400 x g for 8 min.
   h. After the final wash, resuspend the pellet in complete RPMI at 10^4 cells/ml. Do not allow the target cells to stand; proceed quickly to the CML Assay.
5. **Setting Up the Cell-Mediated Lympholysis Assay**
   a. Set up 3 plates, one containing 100 µl per well of ^{51}Cr labelled autologous target cells, one containing 100 µl per well of ^{51}Cr labelled specific allogeneic target cells, and one containing 100 µl per well of ^{51}Cr labelled allogeneic pool target cells (See Figure 1). To quadruplicate wells of each add the following:

–100 µl Medium alone (to obtain the value for spontaneously released ^{51}Cr during the 4 hr incubation).
–100 µl of TX100 (to obtain the value for maximum ^{51}Cr release during the 4 hr incubation period).
–100 µl specific effector cells (stimulated with specific allogeneic cells) at an effector to target ratio (E: T ratio) of 100:1, 50:1, 25:1 and 12.5:1.
–100 µl nonspecific effector cells (stimulated with pooled allogeneic cells) at an effector to target ratio (E: T ratio) of 100:1, 50:1, 25:1, and 12.5:1.
–100 µl control effector cells (responder cells that have been cultured with autologous cells) at an effector to target ratio (E: T ratio) of 100:1, 50:1, 25:1 and 12.5:1.

b. Centrifuge the plates for 30 sec at 250 x g to promote cell/cell contact, then incubate for 4 hrs at 37° C, 5% CO_2 and 95% humidity.
c. Centrifuge the plates for 5 min at 500 x g, then transfer 100 µl of the cell-free supernatant medium to counting tubes.
d. Count samples directly in a gamma-emission counter, or add scintillation fluid and count in a beta-emission scintillation counter.

Interpretation

1. **Controls**
   a. In order to determine if the stimulator cells are adequate for stimulating effector cell production, it may be useful to test an additional responder cell known to be HLA disparate from the test allogeneic stimulator cell. If the stimulator cells are not capable of stimulating the HLA disparate responder to produce CTLs, then the assay is invalid and must be repeated using fresh cells.
   b. The allogeneic pool should control for the ability of the responder cells to respond.
2. **Poor Labelling**
   If labelling is inadequate it may be due to several possibilities.
   a. ^{51}Cr decayed beyond usefulness. Half-life is 27.7 days. Adjustments must be made for decay when labelling (see Table I). ^{51}Cr that is >30 days beyond assay date may not provide adequate labelling.
   b. While it may be possible to use unstimulated cells for targets, unstimulated target cells do not optimally label with ^{51}Cr. Be sure to follow procedure for target cell generation prior to target cell labelling. It will be necessary to determine the desired concentration of PHA for optimum target cell generation.
   c. If cells do not label well, and above suggestions are not appropriate, try adding label directly to the dry cell pellet. This may result in a higher labelling efficiency.

Calculations

Calculation of ^{51}Cr Volume Needed

The volume of ^{51}Cr needed for labelling is calculated by determining the original volume of the solution containing the desired number of µCi ^{51}Cr and dividing by the decay factor from the decay table (Table I). The appropriate decay factor is read by using the number of days past the calibration date.

Volume of ^{51}Cr *needed at initial concentration ÷ Decay factor (see decay tables) = # ml* ^{51}Cr

EX. Calibration date: 2/19/93
Initial activity: 1.0 mCi/ml
Date of use: 2/29/93
Days past calibration: 12
Decay factor (From Table I): 0.741
mCi needed: 0.150 mCi (150 mCi)

150 *µl* ÷ 0.741 = 202 *µl = Volume of solution needed for* 150 *µCi* ^{51}Cr

Calculation of Specific Lysis

1. Obtain the mean or median from the replicate wells.
2. Calculate the % specific lysis for each E:T ratio using the following equation:
3. % Specific Lysis should be determined and reported at all E:T ratios tested.

% Specific lysis = [(CPM of sample – CPM spontaneous release) ÷ (CPM maximum – CPM spontaneous release)] x 100

Calculation of Lytic Units (LU)

A lytic unit (LU) is defined as the number of effector cells needed to produce a standard percent of the maximum ^{51}Cr release. To calculate lytic units, a curve should be plotted from the % cytotoxicity vs the log effector:target ratio. The full curve is sigmoidal, but it may be possible to determine the E:T ratio which gives 20% cytotoxicity from the linear portion of the curve. Twenty percent is commonly used as the percent of maximum release for the definition of the lytic unit (LU_{20}). This ratio is designated $E:T_{20}$.

$LU_{20}/10^7$ effector cells can be determined as below:

$$\#\ LU_{20}/10^7\ effector\ cells = 10^7\ effector\ cells \div (E:T_{20} \times \#\ target\ cells)$$

Ex. $E:T_{20} = 15:1$
target cells/well = 5×10^3

$$\#\ LU_{20}/10^7\ effector\ cells = 10^7 \div [(15) \times (5 \times 10^3)] = 133\ LU$$

The calculation of the $E:T_{20}$ requires several assumptions to be valid across a number of samples.[7] The first is that the linear portions of the curves used for determination of the $E:T_{20}$ are parallel. The second is that the $E:T_{20}$ falls into the linear part of the curve. Since these criteria are not always met at a single E:T ratio, there have been several attempts to modify the equation to correct for this error.[7,8] If lytic units are to be used, these corrected equations may provide a better estimate of the relative NK activity than the uncorrected equation or specific lysis. If it is necessary to use lytic units, refer to references 7 and 8.

References

1. Whiteside TL, Herberman RB: The role of natural killer cells in human disease. Clin Immunol Immunopathol 53:1, 1989.
2. Whiteside TL, Rinaldo CR, Herberman RB: Cytolytic cell functions. In: *Manual of Clinical Immunology*, 4th Edition (NR Rose, EC de Macario, JL Fahey, H Friedman, GM Penn, eds.), p 220, American Society for Microbiology, Washington DC, 1992.
3. Ewel CH, Kuhns DB, Keller JR, Reading JP, Kopp WC: Clinical monitoring of immune and hematopoietic function. In: *Manual of Clinical Immunology*, 4th Edition (NR Rose, EC de Macario, JL Fahey, H Friedman, GM Penn, eds.), p 923, American Society for Microbiology, Washington DC, 1992.
4. Breur-Vriesendorp BS, Vingerhoed J, Schaasberg WP, Ivanyi P. Variations in the T-cell repertoire against HLA antigens in humans. Human Immunol 27:1, 1990.
5. Ortaldo JR, Herberman RB: Heterogeneity of natural killer cells. Adv Immunol 2:359, 1984.
6. Whiteside TL, Bryant J, Day R, Herberman RB: Natural killer cytotoxicity in the diagnosis of immune dysfunction: Criteria for a reproducible assay. J Clin Lab Anal 4:102, 1990.
7. Bryant J, Day R, Whiteside TL, Herberman RB. Calculations of lytic units for the expression of cell-mediated cytotoxicity. J Immunological Meth 146:91, 1992.
8. Pross HF, Baines MG, Rubin P, Schragge P, Patterson MS: Spontaneous human lymphocyte-mediated cytotoxicity against tumor target cells. IX. The quantitation of natural killer cell activity. J Clin Immunol 1:51, 1981.
9. Beatty P: The induction and assay of human cytotoxic T lymphocytes *in vitro*. In: *ASHI Laboratory Manual*, 2nd Edition (Zachary AA and Teresi GA, eds), p 399, American Society for Histocompatibility and Immunogenetics, Lenexa, KS, 1990.

Plate #1: 1 x 10^3 autologous target cells per well			
	Specific Allogeneic Effector Cells	**Pooled Allogeneic Effector Cells**	**Autologous Effector Cells**
100:1	O O O O	O O O O	O O O O
50:1	O O O O	O O O O	O O O O
25:1	O O O O	O O O O	O O O O
12.5:1	O O O O	O O O O	O O O O
	Medium only	TX 100 only	
	O O O O	O O O O	

Plate #2: 1 x 10^3 pooled allogeneic target cells per well			
	Specific Allogeneic Effector Cells	**Pooled Allogeneic Effector Cells**	**Autologous Effector Cells**
100:1	O O O O	O O O O	O O O O
50:1	O O O O	O O O O	O O O O
25:1	O O O O	O O O O	O O O O
12.5:1	O O O O	O O O O	O O O O
	Medium only	TX 100 only	
	O O O O	O O O O	

Plate #3: 1 x 10^3 aspecific allogeneic target cells per well			
	Specific Allogeneic Effector Cells	**Pooled Allogeneic Effector Cells**	**Autologous Effector Cells**
100:1	O O O O	O O O O	O O O O
50:1	O O O O	O O O O	O O O O
25:1	O O O O	O O O O	O O O O
12.5:1	O O O O	O O O O	O O O O
	Medium only	TX 100 only	
	O O O O	O O O O	

Figure 1.

The Quality Assurance / Improvement Program

Deborah Crowe

Overview

The QA/QI program is established in the laboratory to ensure quality in testing for all phases of pre-analytical, analytical, and post-analytical procedures. The laboratory must have a written protocol which addresses how quality will be assessed and monitored for each of these areas. The JCAHO reference data has defined ten basic steps involved in QA monitoring and evaluation:

1. Assign Responsibility
2. Delineate Scope of Care
3. Identify Important Aspects of Care
4. Identify Indicators of Quality
5. Establish Thresholds for Evaluation
6. Collect and Organize Data
7. Evaluate Care
8. Take Action to Solve Problems
9. Assess the Actions and Document Improvement
10. Communicate Relevant Information to the Organization-Wide QA Program

A. Assign Responsibility

The Laboratory Director has overall responsibility for the Quality Assurance Program. However, to ensure quality, the Director must rely on key laboratory personnel to help implement and monitor compliance to QA policies. The QA manual should indicate all key personnel and the responsibilities assigned to each in evaluating and monitoring the indicators for quality. A Quality Assurance Committee will be needed to review QA reports on a quarterly basis and to evaluate the effectiveness of corrective actions.

1. QA Committee – Director, Lab Manager, Supervisors, department representatives.
   a. Evaluate QA needs
   b. Write general QA policies
   c. Monitor QA indicators
   d. Review corrective actions
   e. Assess effectiveness of corrective actions
   f. Present summary of QA report to entire staff
2. Lab Supervisors / Director
   a. Write specific departmental QA policies
   b. Determine QA indicators to be monitored
   c. Compile data from QA indicators
   d. Prepare Quarterly QA report for the department
   e. Review Reagent QC and Maintenance logs periodically
   f. Provide proper training for new employees and documentation of training for new methodologies
3. Laboratory Staff
   a. Document all problems as they occur
   b. Report accurate and timely results
   c. Identify and correct reporting problems
   d. Performance of quality control as required for each procedure
4. Laboratory Director
   a. Review all proficiency testing before submission
   b. Review all proficiency test results when received
   c. Determine appropriate corrective actions when needed
   d. Review Quarterly and Annual QA summary reports.
   e. Ensure that all aspects of the QA program are functioning as intended.
   f. Ensure employee competence

Each department should provide a list of the tests performed and the clinical use for the test. This will provide the basis for identifying the most important indicators of quality that will be monitored as part of the QA program.

B. Identify Important Aspects of Care

Each department must identify the areas most prone to problems and those most likely to adversely affect accuracy of testing or patient care. For example, proper collection, quality testing practices, and good communication of results to the transplant team may be important aspects.

C. Identify Indicators of Quality

For each important aspect of care, a well-defined and measurable indicator of quality must be identified. For example, for the proper collection of sample, one might monitor the number of rejected samples and the reasons for rejection. For quality testing, one might want to monitor QC failures, reagent problems, equipment problems, and performance on proficiency testing. The indicators should be objective and should help direct attention to potential problems or opportunities for improvement. A partial list of the CLIA '88 quality assurance monitors include:

- Test requisition data for completeness
- Test requisition data for relevance and necessity
- Appropriateness of criteria for specimen rejection
- The use of specimen rejection criteria
- The completeness, usefulness and accuracy of test report information necessary for the interpretation of test results.
- The ability to interpret of test results
- The timeliness of reporting test results
- Guidelines for prioritization of tests
- The accuracy and reliability of test reporting systems
- Record storage and retrieval systems
- Quality control remedial actions for effectiveness
- Corrective actions taken for errors detected in reported results
- Corrective action taken for control problems
- The effectiveness of corrective actions taken for any unacceptable proficiency testing result
- The accuracy and reliability of test systems not included in approved proficiency testing programs
- Patient test results that are inconsistent with existing clinical and laboratory data
- The effectiveness of policies and procedures for assuring employee competency
- Documentation of problems and complaints

D. Establish Thresholds for Evaluation

For each indicator, a threshold is established at which intensive evaluation of the problem is triggered. The threshold established is usually dependent on the number of tests or samples being handled. Some critical indicators may warrant 100% compliance and QA review of the variance will result from any failure.

E. Collect and Organize Data

Forms are useful to document problems, corrective actions, proficiency test misses, etc. Some labs are going a step further by entering the information into a database. This allows one to easily sort and monitor the types of problems encountered each quarter. Appropriate staff should be identified to collect the data needed for the QA report. Data should be organized so that can be easily evaluated and compared to the established thresholds for compliance.

F. Evaluate Care

For a laboratory, this refers to the quality of testing which may affect patient care. The QA committee reviews the compiled data and determines if there are any trends or patterns that may indicate a possible problem area. For example, one might see a larger number of amended reports or rejected samples compared to last quarter. An increase in turnaround time may indicate the need for additional personnel or may be related to equipment problems documented for this quarter.

G. Take Action to Solve Problem

When the threshold for a quality indicator is exceeded, members of the QA committee should examine the problem and determine if appropriate corrective actions have been taken. They should attempt to identify the cause of the problem and to provide insight or suggestions for improvement.

H. Assess the Actions and Document Improvement

The effectiveness of the corrective actions must also be monitored. If improvement is not evident by the next quarterly report, additional corrective actions must be implemented.

I. Communicate Relevant Information to the Organization-Wide Quality Assurance Program

Findings from and conclusions of monitoring and evaluation, including actions taken to solve problems and improve care, should be documented and reported through the established channels of communication. The QA summary report should be made available to all staff members and discussed at Lab meetings.

The Quality Assurance Manual

The laboratory's Quality Assurance manual should give general guidelines for maintaining quality in laboratory testing. The manual can serve as a means to organize in one place much of the information required for accreditation. The different aspects of the laboratory QA program are grouped as Pre-Analytical, Analytical, or Post-Analytical Components. Each important aspect of laboratory performance is identified and the following information is specified for each:

Goal
QA indicators
How indicator will be monitored
Evaluation – threshold for compliance and follow-up actions

The QA Manual contains the general policies for how the different components of the QA program are to be carried out. The more specific procedures and data collected are usually kept elsewhere (ex. Reagent QC, Maintenance Records,

Procedure manual). The QA manual indicates how the laboratory is to monitor QA issues. The following outline includes the major components that should be included in a QA Program.

A. Pre-Analytical

1. General Laboratory
   a. Organizational Chart – responsible persons
   b. Plan for Director Coverage
   c. Emergency Notification Plan
   d. Description of Laboratory Space
   e. List of Services Provided and Turnaround times
   f. Accreditations and Licensures
2. Personnel
   a. Job Descriptions
   b. Employee Orientation Program
      1. Risk Management Policies
      2. Disaster Plan
      3. Infectious Control and TB plan
      4. MSDS / Chemical Hygiene Plan
      5. Safety Issues and Universal Precautions
      6. Personal protective Equipment (PPE)
      7. HIV Post-Exposure Prophylaxis (PEP) Program
      8. Drug Testing policy
   c. Employee Training Program
      1. Training provided for job requirements per job description, safety, computer, personal development, and quality.
      2. Documentation of training steps
         - Read procedure in SOP
         - Watch procedure by trained technologist
         - Perform with supervision
         - Perform alone
         - Final approval by Director / Technical Supervisor
         - Documentation of training and competence
   d. Personnel Evaluation
      1. Performance Appraisal
         - Initially assessed after six months and annually thereafter.
         - Based on job accountabilities, responsibilities, goals and pre-defined measures
      2. Competency Assessment – annually
         - Direct observation of test performance
         - Monitoring the recording and reporting of results
         - Review of worksheets and QC records
         - Performance on internal and external proficiency
         - Performance of maintenance and function checks
         - Assessment of problem solving skills
         - Re-training initiated when indicated
      3. Continuing Education
         - Staff development provided to meet individual needs, regulatory and accreditation requirements, and the changing needs of the laboratory
         - Documentation of continuing education is maintained.
   e. Personnel Files
      1. Documents contained in Personnel File
         - Resume
         - Documentation of Education and/or Training
         - Licenses
         - Copy of Certifications (ex. CHT, CHS)
         - Signed Job Description
         - Signed Orientation Checklist
         - Performance Appraisals
         - Competency Checks
         - Incident reports
         - Technical Upgrades
         - Documentation of Continuing Education
      2. Review files annually to document that they contain all required forms. Check that licenses, certifications, performance appraisals, competency checks, CEUs, etc. are up-to-date.

3. Sample Acquisition – must have written criteria
   a. Sample requirements
      1. All samples must be individually labeled with patient's name or other unique identification number and date drawn.
      2. A test requisition should accompany the sample. If not, testing may begin with an oral order from the physician, but a requisition must be received within 48 hours.
      3. The specimen integrity must be preserved (ex. transit time not too long, proper temperature maintained, excessive hemolysis avoided, etc.).
      4. There must be sufficient quantity of sample for the assay.
      5. There must be compliance with proper specimen collection (correct tube, temperature, etc.).
   b. Requisition requirements
      1. The requisition should include:
         Patient ID
         Name and facility of requesting physician
         Date of specimen collection
         Time of collection, if pertinent to test
         Source of specimen
      2. The name and number on the sample vial must match that on the Request form.
      3. The Requisition should contain pertinent medical history, if available.
   c. Shipping requirements
      1. Packing instructions
      2. Storage conditions
      3. Transit time required

B. Analytical

1. Procedure Manual
   a. Policy for Review of Procedure Manual
      - Must be reviewed annually by Director
      - Recommended that testing personnel review annually and participate in updating
   b. Policy for Updating the Procedure Manual
      - Structure to link policies and procedures
        If there is a policy to have a written protocol, then the protocol must appear in the procedure manual.
      - Process to ensure uniformity of SOP and forms
        Control of document versions and effective dates
        – Utilize footer for name of procedure, version date, and page number
        – Use History of Method form to document changes to procedure and date change was made. Should be signed by Director and kept at the end of each procedure. The latest date on this form should correlate with the date in the footer of the procedure.
      - Archive old procedures
        – Remove old procedures or pages which have been changed. Write Date retired or replaced on procedure.
        – Keep old procedures for a minimum of 2 years.
   c. Validation of New Procedures
      All new procedures or modification to procedures must be validated by performing parallel studies or optimization studies. (see Quality Assurance, Chapter 2)
2. Quality Control Program (see Quality Assurance, Chapter 3)
   a. QC protocols for test methods
   b. Reagent QC
   c. Equipment Maintenance
      - Calibration and preventive maintenance in accordance with manufacturer's recommendations, regulatory requirements, and accreditation standards.
      - Complete documentation of equipment identity, results of scheduled calibrations, actions taken, and disposition of equipment is maintained.
      - Defective equipment is identified, controlled, and repaired or replaced.
3. Proficiency Testing
   a. Internal Proficiency – Tech-to-Tech comparisons
   b. External Proficiency
   c. Designation of testing personnel
   d. Review of Proficiency testing and corrective action
   e. Comparison of testing done at different testing sites
   f. Comparison of testing done by different methodologies
4. Review of Results – Correlation with Patient Information
   The laboratory must have a system in place to verify results and to ensure that the results obtained correlate with known patient information.
5. Specimen Referral
   The laboratory must list approved laboratories used for specimen referral. Copies of the accreditation of send-out labs must be kept on file. Any problems with the send-out lab must be documented.

C. Post-Analytical

1. Reporting Results – need written policy for each of the following
   a. Required Information – sample date, test date, lab #, name, results, reference range, interpretation
   b. Generation of Reports
   c. Verification of Reports
   d. Amended Reports
2. Records
   a. Storage of Records – written policies needed
   b. Confidentiality Statement
      Written confidentiality statement
      List of authorized individuals to whom results may be given over the phone
3. Policy for handling of discrepant results
   a. Discrepancies between laboratories
   b. Discrepancies between methodologies
4. Interaction with the Transplant Program and other Clients
5. Quality Improvement
   a. Review and Update of Policies
   b. Problem Identification and Corrective Actions
   c. Evaluation Thresholds
   d. Effectiveness of Corrective Actions
   e. External Inspections
   f. Communication with Staff

QA Forms

The laboratory must maintain a mechanism to document and investigate events which have a potential to affect quality or safety. Forms are very important to document QA problems and corrective actions. Each quarter, the forms are collected, sorted, and the information is recorded on the QA report. The following types of forms may be used to document problems and variances in the laboratory. Samples are included at the end of this section.

A. Problem Resolution Form
This form should be used to document any problem, no matter how minor or serious. It can be used to document problems within the lab, with a client, with the transplant program, OPO, etc. The use of these forms should be encouraged and should become part of the laboratory's routine practice. This form is used to document specimen problems, processing problems, QC problems, computer problems, or client complaints.

B. Incident Report
This form is used for more serious problems that could have been avoided if the laboratory polices had been followed. These reports must have corrective actions documented. Depending on the nature of the problem, a copy of the incident report may be placed in an employee's personnel file.

C. Equipment Failure Report
This report form is used to document instrument malfunctions and corrective actions and/or repairs.

D. Amend Report
This form is used to document that a report was changed. The reasons for the change are explained and corrective actions (if needed) are documented.

E. Proficiency Testing Corrective Action
This form is used to document misses on external proficiency testing. The results are re-evaluated and the possible problem is described with appropriate corrective actions.

The QA Report

The laboratory must maintain documentation of all quality assurance activities, including problems identified and corrective actions taken. A QA report provides a summary of all QA activity and provides a way to detect problems or trends that need further consideration. An accurate and comprehensive QA Report is vital to keeping both the Director and the Staff informed of potential problems so that a concerted effort can be made to solve them.

A major emphasis of current quality assurance standards is that the QA program be designed to effectively evaluate the QA policies and compliance with the policies. Revision of policies and procedures may be warranted based upon the results of the evaluations.

A. Frequency of QA Reporting
At least quarterly, data should be compiled on a QA report. Most problems and incidents should already be documented and on file. An example of a QA report is found at the end of this section, but many similar formats may be used. The results should be made available to the entire staff and is usually discussed at a lab meeting.

B. Safety Inspection
Part of the Risk Management Program requires that routine safety inspections be performed. These are usually done each month and included with the QA report.

C. QA Committee

All problems are reviewed by the QA committee and assessed for need for follow-up actions. Often, it may be difficult to determine if the corrective action was appropriate and the QA committee may want to re-address the problem at the next meeting to verify that the corrective action was effective in solving the problem. If not, additional corrective actions may be needed.

1. It is recommended that a log events be maintained to ensure that the proper steps in resolving a problem are taken.
2. Results of current assessments are compared to previous results.
3. Trend analysis of incidents, errors, and accidents is performed to aid in prioritizing process improvement efforts.
4. Follow-up is performed to determine effectiveness of corrective actions.

Process Improvement – Utilization of QA Data

Purpose: To define a process that can objectively measure the laboratory's level of performance, identify areas where performance can be improved, provide information that will help set priorities for improvement, offer ideas for improvement, and determine whether corrective actions actually resulted in improvement.

Procedure:

1. The Supervisor collects the QA data and summarizes the information on the quarterly QA report.
2. The QI Committee reviews the summary and looks for any trends in the data when compared to last quarter results.
3. The QI Committee will prioritize the problems that require follow-up action.
4. The QI Committee will present findings to appropriate Supervisor who will develop a team to address the problem.
5. An action plan is developed and a designated team member will implement the plan and collect data.
6. The results are reported to the QI committee.
7. The QI committee analyzes the results and determines if the improvement action was successful.
8. If the action was successful, policies and/or procedures are updated to implement the action as standard procedure.
9. If the action was unsuccessful in promoting a positive result, another action plan is developed and the process is repeated.

Benefits of a Good Quality Assurance Program

1. Provides a means whereby all members of the Laboratory from Director to Technologist can have a clear understanding of how the laboratory is performing and can identify problem areas.
2. Provides objective evaluation of problems which can be presented to management to support need for additional staff, new equipment, etc. to correct the problems.
3. The information can be used to further improve the operation of the laboratory.
4. The information can be used when discussing problems with clients. For example, if 90% of rejected samples came from one client, then this could be used to discuss the problem with that client to convince them to try to solve the problem on their end.
5. A strong QA program is essential in protecting the laboratory from legal implications of poor quality in testing. When litigation occurs, the laboratory must have adequate documentation of all actions and problems that may affect testing quality.
6. QA information may help management address issues regarding problem personnel. Proper documentation in personnel appraisals, competency checks, and incident reports are essential in protecting the laboratory if an employee is dismissed for poor performance.

References

1. DCI Risk Management and QA Program, Nashville, TN.
2. Standards for Histocompatibility Testing; American Society for Histocompatibility and Immunogenetics; March 1994.
3. CLIA '88 – Clinical Laboratory Improvement Act; Federal Register 57(40):70001, 1992
4. DCI Laboratory Policy Manual; Nashville, TN
5. LSU Medical Center- Shreveport; QA Manual
6. Bowman-Gray HLA Quality Assurance Program
7. ASHI Laboratory Manual, 3nd Edition. 1994. Ed. A. Nikaein. Ch. VI. Quality Controls
8. Metz, SJ. Quality Assurance in the Histocompatibility Laboratory. In Tissue Typing Reference Manual. Southeastern Organ Procurement Foundation (SEOPF). Richmond, 1993: Ch C.31 20-1 to 21-14.

PROBLEM RESOLUTION FORM

Quality Assurance, Assessment, Control and Improvement Program

Date:

Type of Problem:

Specimen Problem	**Processing Problem**	**Quality Control Variance**
Collection	Accessioning	Controls out of range
Labeling	Sample mix-up	Reagent Problem
Shipping	Transcription error	Instrument Problem
Integrity	Lab Accident	Technical Problem
Volume	Reporting error	Other
Requisition	Interpretation error	Computer Problem
Other	Other	Client Complaint

___Specimen recollection ordered
___Sample verification required
___Test cancelled

Description of Problem:
Attach any other explanatory documents to this form

Corrective Action:

Problem reported to: **Time:** **Tech:**

Reviewed by:_____ **Date:**_____

Follow-up by Quality Assurance Officer:

Comments:

	Yes	No	N/A
Presented at QA meeting			
Needs follow-up			
Problem Corrected			
Interdepartmental notification			

Signature:_____ Date:_____

INCIDENT REPORT

Documentation of laboratory incidents that may affect safety or patient results

Date of Incident:

Nature of Incident: (Circle) Laboratory Quality Safety Client Relations
Other:

Employee Involved:

Seriousness of Incident: (circle) Serious Moderate Minor

Description of Incident:

Corrective Action: (steps taken to prevent re-occurrence)

Proper Authorities notified: _____

Reviewed by:

Supervisor _____ Date: _____

Lab Manager _____ Date: _____

Lab Director _____ Date: _____

QA Committee Review: _____ Date: _____

Laboratory Name, Address

EQUIPMENT FAILURE REPORT

Name of Instrument: _____

Manufacturer: _____

Under Warranty: ____Yes _____No **Service Contract:** _____Yes _____No

Description of Problem:

Service Required: _____Yes _____No **Cost of Repair:** _____

Corrective Actions:

Reviewed by:

Supervisor: _____ Date: _____

Lab Manager: _____ Date: _____

Director: _____ Date: _____

QA Review: _____ Date: _____

AMEND REPORT

Documentation of Error Correction

Patient Name:	_____	Lab No:	_____
Referring Facility:	_____	Department:	_____
Date Error Occurred:	_____	Person involved:	_____
Date Error Found:	_____	Error Reported by:	_____
Date Error Corrected:	_____	Corrected by:	_____
Corrected Report sent:	Yes / No	Date Sent:	_____
Authorized Person notified:	Yes / No	Person Notified:	_____

Description of Error:

Note: Attach copy of Incorrect and corrected report; Must indicate "corrected report". Keep copies for department. Send Amend form and reports to Supervisor and Lab manager for review and then to QA Coordinator for forwarding to Lab Director for review and signature.

Nature of Error: (Circle)
Serious (affected patient care) *Moderate* (could have affected patient care)

Minor (not used in patient care or correction involved update of previous result based on more information or family studies)

Corrective Action: (steps taken to prevent re-occurrence of problem)

Reviewed by:

Lab Manager: _____ Date: _____

Director: _____ Date: _____

QA Review: _____ Date: _____

Laboratory Name, Address

PROFICIENCY TEST CORRECTIVE ACTION

Survey: _____ Sample ID: _____
Date Tested: _____ Tech: _____
Consensus Result: _____
Laboratory Results: _____

Possible Problem:

Corrective Actions:

Reviewed by:

Supervisor: _____ Date: _____
Lab Manager: _____ Date: _____
Director: _____ Date: _____
QA Review: _____ Date: _____

Laboratory Name, Address

Laboratory Name
Address

QUALITY ASSURANCE REPORT

Year: _____ Quarter: 1st 2nd 3rd 4th

Date of Report:_____

Reviewed by: _____

Fire Drill: Yes / No Date:_____

Safety Inspection: Yes / No Date:_____ Tech: _____

I. Pre-Analytical

		Monthly Tally			
Indicators	**Threshold**				**Total**
Specimen Problem					
Collection Problem	<5				
Mislabeled Sample	<2				
Sample Integrity	<5				
Sample Volume	<5				
Shipping Problem	<5				
Requisition with required information (review 20 requisitions)	100%				
Misc. Problem Resolution forms (attach copies to report)	<5				

QA REPORT (page 2)

____ Quarter

II. Analytical

		Monthly Tally			
Indicators	**Threshold**				**Total**
Processing Problems					
Accessioning Problem	<2				
Sample Mix-up	0				
Transcription Error	0				
Lab Accident	<2				
Tech Error	<2				
Interpretation Error	<2				
Misc. Problem Resolution forms	<5				
Turnaround Time met (review 20 reports)	>95%				
External Proficiency (% correct)	>95%				
Internal Proficiency (% correct)	>90%				
No. of **QC** corrective actions	<2				
No. of **Reagent** corrective actions	<2				
No. of **Equipment Maintenance** corrective actions	<2				

III. Post-Analytical

Indicators	**Expected**				**Total**
Reports contain all required information (review 20 random reports)	>95%				
No. of **Amended Reports** (Attach copies)	<2				
No. of **Client Complaints** (Attach copies of report)	<2				
No. **Incident Reports** – due to noncompliance to lab policies	<2				
Computer Problems					

Comments / Follow-up

QA COMPLIANCE DOCUMENTATION

To be completed monthly by Director or Supervisor responsible for monitoring compliance to QA policies and procedures.

Prepared By: _____ Date: _____

1. Is there evidence that Problem Resolution Forms and other QA forms are being used to document variances in the laboratory?
   Yes / No

2. Have there been any Incidents due to failure to follow lab policy this month? _____

   If Yes, was there proper documentation? _____
   Corrective Action? _____
   Follow-up needed? _____

3. Has all Equipment Preventive Maintenance been performed according to schedule? _____

4. Has all reagent QC been documented and reviewed? _____

Documentation of Misc. QA policies:

Item	Frequency	Completed? Y/N	By whom?	Date
Method Comparison	January and July			
Storage of Records for 2 years; (check availability of 5 records that are 1-2 years old)	November			
Internal Proficiency – tech checks	Monthly			
Employee Competence	December			

Ideas for tasks which can be made easier/safer by changing a process or re-designing the task:

_____
_____
_____
_____

Miscellaneous Observations and Comments:

_____
_____
_____
_____

Quality Assurance of Information / Data in the Laboratory: New Test Validation, Patient Test Management, Computerization, and Laboratory Data Maintenance

Lori Dombrausky Osowski

Principle

The laboratory must incorporate a component into the Quality Assurance Program for data management issues including new test validation, patient test management, laboratory data maintenance and computerization. This chapter will discuss important issues for the laboratory personnel to maintain in order to have a viable and meaningful Quality Assurance Program, but by no means encompasses all future issues that may be identified as important to monitor. As new laboratory methods, software and new information systems become available in the future, a Quality Assurance Program must grow and mature with the technology.

I. New Test Validation

All new and revised tests must be validated prior to implementation by the laboratory. All new procedures must be approved by the Director / Technical Supervisor. All technologists performing the new procedure must document that they have been trained and are competent to perform the new or modified procedure. The following components must be completed and approved:

A. Parallel Testing

1. Parallel testing is performed on materials with another validated method or with another accredited laboratory performing the same test.
2. A blind study is preferred, in which the laboratory is unaware of the results of the test samples prior to testing.
3. The amount of parallel testing needed may be designated by an accrediting agency or determined by the lab itself, depending on the amount of new testing that is being brought into the laboratory.

B. Reproducibility Studies

1. Intra-run reproducibility – documentation that the same answer is obtained when the sample is tested in duplicate on the same run.
2. Inter-run reproducibility – documentation that the same answer is obtained when a sample is tested on two different runs.

C. SOP (Standard Operating Procedure)

The new procedure must be written and incorporated into the department's procedure manual. Old procedures that have been replaced with a modified version must be removed from the procedure manual. The date the old procedure was retired or replaced is written on the old procedure and the old version is saved for two years.

D. Documentation of Training

1. A training guide (if necessary) is established.
   If this is a new procedure, a module may need to be added to the training manual to incorporate the tasks necessary to master in order to perform the new procedure.
2. Documentation of training of all personnel who will be performing the new procedure.
3. Quality control, equipment calibration and maintenance must be established and implemented.
   a. Proper QC measures must be established for the procedure and these must be included in the SOP for the procedure.
   b. QC forms may be needed for the procedure for proper documentation that adequate QC was performed and was within tolerance limits set for the procedure.
   c. Equipment calibration procedures must be included in the SOP and must be documented to have been performed. Tolerance limits for accepting or rejecting calibration results must be established.

d. Preventive maintenance procedures for equipment used in the test must be established and included in the SOP or Equipment Maintenance manual. Forms may be needed to document that preventive maintenance was done according to the schedule established in the laboratory.
e. The impact of any internal and external operations must be assessed. For example, if incubation conditions are changed, one must validate the effect of the change on test results after proper parallel studies have been performed.
f. After the new test is in place and is operating as an SOP, then the process must be monitored at intervals to determine if the new test is effective as implemented to attain the laboratory's initial goal.
g. Flow charts or checklists may also be helpful to help aid in this process (see Figure1).

Figure 1.

Test Validation Checklist		
TASK	**BY**	**DATE**
1. Design a validation protocol		
2. Construct a flowchart of the process		
3. Perform Parallel Testing		
4. Write an SOP		
5. Write a training document		
6. Formulate Competency Training Forms		
7. Determine necessary equipment and reagent quality control		
8. Write a quality control SOP		
9. Design QC forms to capture QC data		
10. Determine the necessary preventive maintenance and calibration schedule for equipment		
11. Design a training schedule for the new SOP		
12. Train personnel and document training		
13. Assess effect on internal and external operation processes		
14. Assign or develop any needed system checks		
15. Collect data on the quality indicators (system checks) and monitor performance		
16. Implement any necessary corrective action		
17. Conduct any necessary process improvement activities		
18. Design forms needed to capture any results from the new SOPs		

II. Patient Test Management

Patient test management involves setting protocols and monitoring compliance for patient preparation, specimen collection, labeling of samples, transport of samples, and processing of samples during testing. The laboratory must have written procedures for each of these and all tests must be accompanied by a written request within 30 days.

A. Test Requisitions – must include following:

1. Patient name or other unique identifier
2. Name and address or other suitable identifier for requesting client
3. Tests ordered
4. Date of specimen collection
5. Other relevant information – ex. ethnic group, relationship to recipient, immunization events, drugs
6. Oral requests must be followed by a written request within 30 days.
7. Requisitions must be kept for a minimum of 2 years.

B. Patient Preparation

1. Instructions for special preparation of patient must made available to the client for each test performed in the laboratory. For example, serum for crossmatch must be drawn prior to dialysis, at least 2 weeks after a sensitizing event, or prior to induction immunosuppression.
2. There should be a policy for when pre-scheduling is required for a test. This information needs to be made available to client.

C. Specimen Collection

1. The lab shall have written criteria for sample volume, type of anticoagulant, storage conditions, and transport requirements for each test ordered.
2. The samples for testing in the HLA laboratory must be collected in the appropriate tubes and stored under the correct conditions in order to maintain cell viability during transport and storage.

D. Labeling of Samples

1. The sample must be properly labeled with name and/or identification number and the date drawn. The initials of the phlebotomist should also be on the tube.
2. Criteria for rejecting samples:
   a. Sample unlabeled
   b. Identification of tube and requisition do not match
   c. Poor viability due to improper storage and/or transport
   d. Incorrect tube used for collection
   e. Insufficient quantity to perform test
   f. Tube broken

E. Transport of Specimens

1. Sample tubes must be shipped in special specimen mailing boxes, which are double-lined, and include protective packing to prevent breakage during shipping.
2. A biohazard label must be attached prior to shipping.

F. Processing of Specimens

1. Ensuring Reliable Specimen Identification during Processing
   a. Samples must be properly labeled and match requisition
   b. The sample is given a unique laboratory accessioning number which is used during processing.
   c. The laboratory number is placed on all worksheets and tubes used during testing.
   d. When reading trays, the number appearing on the worksheet and tray are re-checked and matched before recording results.
2. Relationship of Patient Information to Patient Test Results
   a. The results are reviewed by at least two individuals
   b. The results are compared to past results and family typing to ensure that they do not conflict with previous data.
3. Turnaround time is monitored to ensure that results are reported in a timely fashion.
4. Clients must be notified of test changes that affect test outcome or interpretation. SOPs must reflect these changes.
5. There must a mechanism in place to monitor complaints and problems that affect patient test management and clinical consultation available to clients. (See Figure 2)

Figure 2.

Patient Test Management Checklist

TASK	BY	DATE
1. Does the laboratory must have written procedures for patient preparations, specimen collection, labeling and transport?		
2. Are all tests accompanied by a written request within 30 days?		
3. Do test requisitions include: the patient name or other unique identifier, name and address or other suitable identifier for requesting client, the tests to be performed, date of specimen collection, and any additional data relevant and necessary to a specific test, in order to assure timely testing and reporting of results, such as ethnic group, relationship to other family members, immunizing events or drugs?		
4. Are requisitions kept for a minimum of two years?		
5. Are turnaround times monitored to ensure timely reporting of results to clients?		
6. Is a list of test methods, performance specifications and other data that may affect interpretation of results available to clients?		
7. Are clients notified of test changes that affect test outcome or interpretation? Do SOP's reflect changes?		
8. Is there a mechanism in place to monitor complaints and problems that affect patient test management? Is clinical consultation available to clients?		
9. Has an SOP been written for patient test management issues? Is there a written protocol for sample handling during the testing process to ensure that proper identity is maintained?		
10. Are personnel trained properly and training documented?		
11. Assess effect on internal and external operation processes		
12. Assign or develop any needed system checks		
13. Collect data on the quality indicators (system checks) and monitor performance		
14. Implement any necessary corrective action		
15. Conduct any necessary process improvement activities		
16. Design forms needed to capture any results from the new SOPs		

III. Computer Validation Protocol

The laboratory must perform validation and revalidation of computer systems including the associated software, whenever new hardware, new or upgraded software, and new and changed interfaces are implemented.

A. Validation of New Computer Programs

New computer programs must be documented and verified to perform as expected and validated for accuracy after installation.

B. Monitoring of Accuracy of Computer-Assisted Calculations or Interpretations

All applications that perform an analysis that was previously performed manually must be monitored for accuracy on a regular basis to assure correct performance.

C. Dedicated Personnel for Computer Program Maintenance and Upgrades

Access to the computerized systems must be limited to appropriate persons, in order to maintain integrity, security and confidentiality of data.

D. Computer Back-up Systems

There must be tracking capabilities of electronic records and activities, a protocol for backing up data, ability to reissue data electronically and a backup plan for "down time" incidents.

E. Computer Support

Support services for the system must be identified and in place. (See Figure 3)

Figure 3.

Computer Systems Validation Checklist

TASK	BY	DATE
1. Design a validation protocol		
2. Construct a flowchart of the process		
3 Have new programs been documented and verified to perform as expected and validated for accuracy after installation?		
4. Write an SOP		
5. Write a training document		
6. Formulate Competency Training Forms		
7. Is access to the computerized systems limited to appropriate persons in order to maintain integrity, security and confidentiality of data?		
8. Is there a tracking capability for electronic records and activities?		
9 Is there a protocol for backing up data, ability to reissue data electronically and a backup plan for "down time" incidents?		
10. Are there support services for the system identified and in place?		
11. Design QC forms to capture QC data		
12. Design a training schedule for the new SOP		
13. Train personnel and document training		
14. Assess effect on internal and external operation processes		
15. Assign or develop any needed system checks		
16. Collect data on the quality indicators (system checks) and monitor performance		
17. Implement any necessary corrective action		
18. Conduct any necessary process improvement activities		
19. Design forms needed to capture any results from the new SOPs		

IV. Laboratory Data Maintenance

The laboratory must maintain data storage and maintenance, have appropriate access to data, and verify data for accuracy.

A. Laboratory Records

1. The test reports must be delivered promptly to the authorized person(s) .
2. Duplicates of reports should be maintained by the laboratory for minimum two years.
3. Data must be reported in a timely, reliable and confidential manner.
4. Test records must specify the condition and disposition of specimens that do not meet the laboratory's established criteria for specimen acceptability.

5. Requirements for reports
   a. Testing laboratory's name,
   b. Testing laboratory's address
   c. Pertinent test and normal values
   d. Collection date of sample
   e. The unique sample identifier number
   f. Name of individual tested
   g. Date of report
   h. Test results
   i. Test methods (when appropriate)
   j. Appropriate interpretations
   k. Signature of Lab Director or Designee
6. Panic values must be called directly to clients.
7. The lab must maintain confidentiality and security of data.

B. Storage of Records

1. The lab must follow regulations regarding long term storage of records and documents.
2. Records must be kept and readily available for at least two years, but may be longer, depending on which regulatory agencies oversee the laboratory. (See Figure 4)

Figure 4.

Laboratory Data Maintenance

TASK	BY	DATE
1. Maintenance, have appropriate access to data, and verify data for accuracy		
2. Are the test reports delivered promptly to the authorized person(s) and are duplicates of reports maintained by the laboratory for minimum two years?		
3. Does data reported in a timely, reliable and confidential manner?		
4. Do test records specify the condition and disposition of specimens that do not meet the laboratory's established criteria for specimen acceptability?		
5. Does the report must include the testing laboratory's name, address and pertinent test and normal values? Are panic values directly delivered to clients?		
6. Do reports contain: the collection date of sample, the lab's unique identifier, name of individual tested, date of report, test results, test methods and appropriate interpretations and signature of the lab director, or designee?		
7. Does the lab maintain confidentiality and security of data and follow regulations regarding long term storage of records and documents? This time is at least two years, but may be longer, depending on which regulatory agencies oversee the laboratory.		
8. Design an SOP		
9. Train personnel and document training		
10. Assess effect on internal and external operation processes		
11. Assign or develop any needed system checks		
12. Collect data on the quality indicators (system checks) and monitor performance		
13. Implement any necessary corrective action		
14. Conduct any necessary process improvement activities		

References

1. B, *A Model Quality System for the Transfusion Service*, Transfusion Service Quality Assurance Committee, 1997.
2. *Clinical Laboratory Improvement Amendments of 1988, final rule*. Federal Register, 57(40):7001,1992.
3. Cox, F., S. Vaidya and G. Teresi, *Quality Assurance for Serology and Cellular Methods*, ASHI Laboratory Manual, 3rd Edition. VI.9.1
4. ASHI Accreditation Standards, Guidelines and Checklist, March 15,1995.

Facilities and Environment

Geoffrey A. Land

Overview

An integral part of any Quality Assurance or Continued Quality Improvement Program is the assessing of workplace safety. There are several regulatory agencies that routinely monitor laboratory working conditions (HCFA, CDC, JCAHO, OSHA). Moreover, they have determined that employees have a right to know about what hazards or potential hazards will be encountered while performing their jobs and that they must receive this information in their initial training. These agencies further require management to develop action plans to resolve any physical or environmental problems in the workplace, implement the plan, and document the success of their actions by thorough review of the data. Finally, the employee's knowledge of the information must be documented through performance evaluations and competency tests.

To have a viable laboratory safety program, it is not enough to have written policies and procedures. It is necessary to apply these policies and procedures in a consistent evaluative process. This process includes, but is not limited to, the consistent collection of and supervisory review of all environmental data (ambient and testing temperatures, hazardous chemical and biological exposure, etc.). More importantly, values outside defined acceptable ranges must be brought to a supervisor's attention immediately and corrective action must be taken and documented. Results of environmental assessments made by other than laboratory personnel (electrical safety, fire safety, air handling, etc.) must not only be available for review by regulatory agencies and the institution's administration but also for review by the laboratory staff. As with all laboratory documents, environmental assessments should be readily accessible and it is suggested that these materials be collated into a single electronic or paper file/folder.

This chapter describes the various categories of environmental factors to be assessed, specific items within each category, and required or recommended practices for dealing with specific hazards. The factors and their degree of relevant importance or risk will vary among laboratories and over time within a laboratory. As laboratory practices and methods change, so may the environmental hazards, rendering this chapter incomplete. No rules or guidelines can substitute for a commitment to assuring a safe work place.

I. Physical Facilities

A. Space

1. **ASHI Standard C1.000**. *(UNOS C1.100): "Laboratory space must be sufficient so that all procedures can be carried out without crowding to the extent that errors may result."*
   Federal Regulation 493.1204: *The laboratory must provide the space and environmental conditions necessary for conducting the services offered.*

   With that said, there are no hard and fast rules about the amount of space necessary to accomplish all of the tasks implicit in histocompatibility testing and the assurance of quality results. However, inadequate space may cause a variety of serious problems including:
   a. Jostling a nearby worker, causing a spill of hazardous materials
   b. Specimen mix-ups
   c. Sub-optimal test performance
   d. Increased injury risk
   e. Violation of federal, state, and/or local regulations
   f. Demoralization of technical staff and reduced attention to detail
2. A space of approximately 30 square feet per individual is usually adequate for a single task. This space accommodates a 5 ft. x 2 ft. bench, 1 ft. clearance, and a 3 ft. wide aisle. The three feet aisle provides unobstructed space for anyone working behind the individual at this space. However, additional space is necessary for:
   a. test equipment (e.g., microscopes, centrifuges, biosafety hoods, fume hoods, incubators, thermocyclers, computers, water baths);
   b. storage of specimens and reagents at required temperatures;
   c. record storage that provides easy access;
   d. segregation of certain functions (e.g. pre- and post- DNA amplification, specimen handling and paperwork) and certain types of hazardous materials (e.g. radioisotopes, materials that produce toxic fumes, etc.);
   e. storage and disposal of hazardous materials (e.g. human tissues, sharps, radioactive waste, combustibles, etc.);
   f. appropriate numbers and types of safety equipment (e.g., fire extinguishers, eyewash stations, safety showers, fire blankets, hazardous spill kits, etc.); and
   g. storage of personal protective equipment.

B. Extent of Service

1. Lighting must be sufficient to prevent eye fatigue, especially for those tasks requiring pipetting small volumes.
2. Ventilation must be adequate to prevent accumulate of potentially toxic gases (e.g., CO_2, N_2, etc.) and/or volatile toxic chemicals.
3. Facility and equipment temperature verification
   a. Ambient temperature and humidity must be controlled within the range specified for optimal test performance. The ambient temperature must be monitored on a daily basis.
   b. All temperature maintaining equipment (incubators, freezers, refrigerators, water baths, heating blocks, dry baths, thermocyclers, etc.) must be operated at temperatures optimal for their tasks or the storage of each specimen type or reagent used in the laboratory. Temperature ranges should be those defined by the laboratory's procedure manual and/or reagent manufacturer.
   c. Monitoring
      (1) Incubators, refrigerators, and freezers – daily
         (a) Recording thermometers are recommended for incubators, mechanical refrigerators, and freezers. Otherwise, manual temperatures must be recorded with linear or minimum/maximum thermometers that have been calibrated with a National Bureau of Standards thermometer.
         (b) Refrigerators and freezers – should be coupled with audible alarm, which can be heard 24 hours per day
         (c) For CO_2 incubators – temperatures and CO_2 concentration should be monitored daily. The latter should be within ± 1% of the concentration specified in the procedure manual for that task.
      (2) Thermocyclers – monthly, or as needed for discrepant reactions
      (3) Liquid nitrogen – level of LNO_2 monitored at intervals which ensures an adequate level is present at all times. An automated LNO_2 system with recording temperature and on board alarm is recommended. If a Dewar flask is used then, depending upon the rate of evaporation of that particular unit, then monitoring can be as often as daily or once or twice a week.
   d. All temperatures and gas concentrations (CO_2 and LNO_2) are recorded on a form initialed and dated daily by the recording technologist and must be reviewed by the General Supervisor and Director on a monthly basis.
4. The facility must provide for emergency power and backup freezer space, should either or both fail.

C. Mechanical Safety

1. Mechanical safety has to do with the positioning of objects so that they do not inhibit free movement of the employee.
2. Guidelines for preventing some frequent causes of laboratory injuries include:
   a. Eliminate projections that protrude into corridors and work areas (doorknobs, fire extinguishers, sharp edges and floor attachments).
   b. Provide adequate space for movable objects such as drawers, doors, and machinery to operate freely. Place guards and shields on equipment with exposed moving parts, whenever possible and provide warning labels or signs in all other cases.
   c. Supplies must not be stored in corridors and work areas. These present hazards that may cause serious falls, particularly if visibility is reduced by smoke or power failure.
   d. Dangerous reagents or heavy objects must not be stored on high shelves and at least an eighteen inch clearance must be provided between the top shelf or its contents and the ceiling (Note: This height may differ according to local fire or safety regulations).
   e. Chains or other safety strapping must be used to hold heavy tanks such as those used for compressed gases (oxygen, nitrogen, etc.) upright and pressure reducing regulators must be used to limit gas flow.
   f. If engineering or physical plant personnel monitor mechanical safety, copies of any evaluations must be made available to laboratory personnel.
3. Employees should know the locations of all safety equipment, such as spill kits for flammable solvents, fire extinguishers, fire exits, safety showers (the best method of extinguishing burning clothing), and fire blankets, in addition to the person(s) to call when the general safety of the workplace is compromised.

D. Electrical Safety

All employees should have general knowledge of the fundamental principles of electricity and electrical safety. This should include a general understanding of the physiology of electric shock, especially emphasizing how tetany is induced in muscle and how to avoid the electrical current running to ground through the heart. Employees need to know that electricity finds the path of least resistance to ground which, in some instances, may be through the employee's body. They should also understand the importance of grounding equipment properly, avoid overloading electrical outlets, and avoid the use of extension cords.

1. Laboratory electrical hazards represent the combined possibilities of shock, fire, and the release of asphyxiating vapors and gases. For this reason alone, there has to be an ongoing electrical safety program for the facility and its equipment.
   a. The institution's engineering or physical plant personnel usually monitor electrical safety, but it is incumbent on the laboratory staff to be aware of their findings. Consequently, copies of all documents pertaining to electrical safety must be available to the laboratory.
   b. At the least one employee per shift must know the location of the electrical control (panel) box for the laboratory and how to cut off the power supply in an emergency.

2. Suggestions for greatly reducing electrical risks.
   a. *Grounding of appliances* – accounts for most electrical accidents.
      (1) Three-prong plugs – connecting all appliances to outlets.
      (2) Ground fault circuit interrupters – useful with some appliances. They interrupt power in short circuits and can be used to test to see if the grounding circuits of an appliance are satisfactory.
      (3) Do not handle electrical items with wet hands, gloves, feet, or body.
      (4) Initially touch electrical appliances with the back of the hand, otherwise a shock may cause the fingers to curl forward and possibly prolong the contact.
      (5) Computers and other complex equipment using microchips and/or central processing units (flow cytometers, ELISA recording spectrophotometers, thermocyclers, etc.) should be plugged into surge protectors to protect them from power fluctuations. Surge protectors with battery back-ups provide uninterrupted power to crucial pieces of equipment such as computers and CPU driven equipment such as flow cytometers, spectrophotometers, thermocyclers, etc.
   b. *Preventive equipment maintenance* – identifies most developing hazards.
      (1) Examine instruments each time they are used to ensure there is no cord damage and that any necessarily exposed contacts are properly guarded.
      (2) Equipment should be monitored carefully for overheating and should be located far away from combustible materials, if routinely operated at high temperatures.
3. Hospital based laboratories
   a. All electrical equipment associated with a hospital, including hospital based laboratories, must be safety tested in accordance with the *American National Standards Institute/National Fire Protection Association 99 (ANSI/NFPA 99).*
      (1) The standards make no distinction between hospital, patient, or employee owned equipment, all must be safety tested.
      (2) Must be tested at least annually and those records must be on file and reviewed by the management staff.
      (3) All staff responsible for reporting defective equipment.
      (4) Usually done by hospital Biomedical Engineering Department. Records must be made available to the laboratory.
   b. These standards are enforced by the following agencies
      (1) The Joint Commission on Accreditation of Health Care Organizations (JCAHO)
      (2) OSHA
      (3) HCFA
   c. Medical Devices Act – requires that any medical device contributing to serious injury and/or death must be reported to the Food and Drug Administration within 10 days of the incident.
   d. Any occurrence or incident report generated by faulty electrical equipment must be reviewed by the Director and Supervisor and corrective action documented.

E. Fire Safety

1. Three ingredients are needed for fire or combustion: fuel, ignition and oxygen. The opportunities for these factors to combine in the laboratory are more frequent than one would like. Even partial burning which develops only smoke can be as dangerous as fire.
2. The precautions given below limit the fuel, ignition or oxygen sources, and will prevent or reduce the likelihood of having a laboratory fire.
   a. Fuels
      (1) Flammable liquids, such as acetone, xylene or toluene, are a primary source of fuel. (Ignition of a spill from breaking a one-gallon glass bottle can produce ceiling temperatures of nearly 900 degrees F within one minute.)
      (2) Suggested handling
         - Store relatively small amounts in safety containers (no glass) on bench
         - Keep away from electrical heating units
         - Store containers in special air-tight cabinets
         - Limit total quantity on hand.
         - Store only in refrigerators made for flammables.
         - Provide ventilation for flammable gas or vapor.
   b. Ignition sources
      (1) Static electricity or improperly grounded electrical equipment. There is enough static electricity to ignite vapors generated by simply pouring large quantities of solvents from one container to another.
         (a) Prevented by using grounded metal funnel located in a fume hood
         (b) Decreasing the distance through which the solvent is poured, and by keeping at least 50% humidity in areas of solvent transfer.
      (2) Portable heating devices, such as Bunsen burners or propane torches, are also sources that must be controlled, primarily by keeping them away from vapor areas or combustible materials.

c. Oxygen is always present where people work, but concentrated sources are found in oxidizing chemicals, such as nitric or sulfuric acid. Small amounts of fuel or a spark or small flame in the presence of an oxidizer can cause an explosion. Such chemicals should be protected by using bottle carriers and special storage areas.

3. Fire protection measures should include detection systems, employee fire drills, and clearly posted evacuation routes.
   a. The most frequent causes of laboratory fires are carelessness, lack of knowledge, smoking, unattended operations, faulty electrical devices and unsafe environments.
   b. Escape routes must be posted, as required by inspecting agencies and common sense.
   c. Precautions that must be in documented operation
      - Escape route posted
      - Outside assembly area identified for lab
      - Smoke alarm active
      - Alarm system audible
      - Sprinkler system turned on
      - Fire communication procedure identified
      - Drill practices held yearly
      - Escape route uncluttered (60-inch corridors minimum)
      - Emergency lighting available
      - Know when, where, and how to fight a fire
   d. Most of the activities encompassed within Fire Safety are usually the responsibility of physical plant personnel acting in concert with the local fire authorities. Any documents generated during these activities must be available to the laboratory.

F. Thermal Hazards

Thermal hazards include cryogenic solids and fluids, such as dry ice (CO_2), liquid nitrogen (LNO_2), and freon as well as normally functioning gas or electrically heated equipment that can cause skin burns.

1. Technologists working with LNO_2 should use face shields to avoid splashes or projectiles of broken containers that are caused by rapid warming of the LNO_2.
2. Controls for high temperature equipment should be located to avoid contact with the heating source.
3. Suggested precautions for the handling of dry ice
   a. Packaging must prevent pressure build-up by releasing CO_2 gas.
   b. Dry ice weight should appear on the outside of the package
   c. Dry ice must be placed within the secondary packaging.
   d. Secondary packaging must remain unaltered after release of CO_2
   e. Packaging must be able to withstand the temperatures and pressures encountered during transportation, if such were lost.

G. Waste Management

1. All material contaminated with blood must be bagged and labeled as biohazardous waste, and either sterilized before general disposal, incinerated or disposed in accordance with institutional, local, and state policies.
2. Containers must be leakproof and/or contain sufficient absorbent material to contain liquids so that no spills occur.
3. Blood-contaminated sharp instruments and needles must be disposed in containers that can be handled without danger of skin puncture.
4. Final disposition of medical waste must be according to local, state, and Federal regulations.

H. Hazardous Materials Program

The laboratory's Q/A program must also include documentation of adequate and appropriate management of hazardous materials. This includes proper classification, labeling, transportation, and instructions for shipping instructions of hazardous materials as well as reporting of all incidents and accidents incurred during the handling of such agents. The staff must review all documents pertaining to these materials annually.

1. Classes of hazardous materials
   a. Explosives
   b. Gases
   c. Flammable liquids
   d. Flammable solids
   e. Oxidizers
   f. Poisonous materials
   g. Infectious substances
   h. Radioactive materials
   i. Corrosive materials
   j. Dry Ice and other miscellaneous reagents/supplies

   NOTE: Transportation of materials, Chemical Hazards, and Radiation Safety will be discussed in separate sections below.

2. Labeling
   a. **Biohazard wastes:** Transport in containers with BIOHAZARD symbols printed or affixed to them. Commercial trucks are placarded according to the Department of Transportation (DOT) regulations.
   b. **Other hazardous materials:** Must have proper hazard labels placed next to the shipping name on the container. The package must accommodate all labels without having a label wrap around the package face.
   NOTE: Infectious substances (class 6.2) should have the label Class 6, *"Infectious Substance"*
   c. Diagnostic specimens: Requires the OSHA BIOHAZARD label and the following text:
   "Diagnostic specimens – packaged in compliance with IATA Packing Instruction 650."
   Diagnostic samples do not need a DOT label.
   d. All packages: Must conform to OSHA's blood borne pathogen standard for labeling.
3. Information necessary for hazardous exposure program (Chemical and Radiation exposure will be handled in separate sections. See below)
   a. Documentation of all work related accidents, injuries, and illness due to exposure
   b. Problem Resolution or Incident Reports
   c. Follow-up testing (viral serologies, culture, etc.)
   d. HIV considerations:
      (1) Post-exposure detection and prophylaxis program
      (2) Employee counseling
      (3) Permission slip to have putative source(s) tested
   e. Workman's compensation policies relative to exposure
   f. Short and long-term disability expectations
   g. Early return to work program
   h. Medical Leave Act/Disabilities Act policies as they relate to exposure
4. As part of part of any continuing quality assessment program there should be routine, documented monthly safety hazard checks as well as compliance with other departmental Q/A policies. Some items may need no more than an annual review. If these data are collected by another department, they must be made available to the laboratory

I. Transportation of Samples

1. Biological specimens must be packaged in sturdy containers with sufficient surrounding absorbent cushioning material to contain any leakage and double bagged where appropriate.
2. Fully processed blood products have generally been exempted from these requirements, being deemed by the Food and Drug Administration (FDA) as regulated products carrying little or no risk to handlers.
3. The packaging requirements for transporting untested blood products outside of the manufacturer's control requires the use of leak-proof packaging and sufficient absorbent material to contain any leakage.

   NOTE: It is the senders' responsibility to protect the shipper.
   a. Substances must be classified for shipping as described below.
      - Proper shipping name
      - Hazard class – assign only 1 (and subdivision, where applicable)
      - Identification number (see Hazards Material Table)
      - **UN** number – United Nations number, domestic and overseas shipping
      - **NA** number – North American number, US and Canada only
      - Packing group
        – Group I (great danger)
        – Group II (medium danger)
        – Group III (minor danger)
   b. Shipping Papers
      - Name and address of consignee
      - Name and phone number of responsible party
      - Nature and quantity of goods
      - Shipping name, hazard class, Packing group, UN/NA identification number, Packing instruction number
        NOTE: Infectious material have no packing group
      - Quantity of shipment by weight or volume
      - Number of packages and type
      - Indicate overpacking
      - Emergency response information – CDC emergency phone number, if material infectious
      - Name, title, place, date, and signature of person preparing package
      - Shipper's certification
        " I hereby declare that the contents of this consignment are fully and accurately described above by the proper shipping name, and are classified, packaged, marked, and labeled/placarded, and are in all respects in the proper condition for transportation according to the applicable international government regulations"
      - Diagnostic and dry ice shipments aren't restricted and require no shipper's declaration
      - For infectious substances, include under *"Additional Handling Instructions"*:
        Prior arrangements as required by the IATA Dangerous Goods regulations 1/3/3/1 have been made.

4. Material should be completely labeled and contents of package fully disclosed, as in the following examples:
   a. *Infectious substances*
      - Obtain manufacturer's Department of Transportation certification with performance oriented packaging (POP) criteria
      - Special markings necessary for infectious substance packaging
        – "UN" packing symbol
        – Packing type code = 4G
        – Text = Class 6.2/Yr of Mfg.
        – State or country international vehicle code authorizing Mfr. to ship
        – Name of manufacturer
      - Criteria for secondary packaging
        – Non-leak
        – Internal pressure ≤ 13.8 lb/in^2
        – Temperature range -400° C to 550° C
      - Itemized contents list/requisitions between inner and outer containers
      - Shipper's name and telephone number on outside package
   b. *Diagnostic samples*
      - Inner packaging
        – Non-leak
        – Secondary packaging (water tight)
        – Absorbent material between primary and secondary packaging
      - Outer packaging
        – Strength adequate for intended use
        – Withstand 1.2 meter drop and pressure tests
        – 4" Minimum dimension for shipping
      - Packing list/requisitions between primary and secondary container
      - Air shipping must be indicated on package and waybill
      - Labeling
        – Infectious substance, affecting humans
        – "Dry Ice" (when applicable)
        – UN or NA identification number
        – Name and address of consignee and consignor
        – Arrows indication correct "Up" position
        – Name and telephone number responsible party
        – Outer label: "Inner packages comply to prescribed specifications"
        – Total amount of infectious substance (e.g. ≤ 1ml)
        – Information written in English

II. Biologic and Chemical Hazards

A. Biologic Hazards

1. Job description and risk of exposure
   One of the mandates of the Occupational Health and Safety Administration Act (CFR 1910.1000 to end, 1 July, 1997) is to define the relative risk of an employee becoming exposed to a biohazardous agent based upon his/her job description.

   Category 1: Procedures and tasks relative to this job description involve exposure to blood, tissues or body fluids (amniotic fluid, pericardial fluid, peritoneal fluid, pleural fluid, synovial fluid, cerebrospinal fluid, semen, and vaginal secretions) or body fluids grossly contaminated with blood via mucous membrane or skin contact or trauma. Persons in this category are must routinely use personal protective equipment (PPE) such as disposable laboratory coats, gloves, goggles and/or face shields, while performing their tasks.

   Category 2: Routinely does not perform tasks that would lead to exposure to contaminated fluids and tissues but, upon occasion may be asked to perform Category 1 activities. Must wear PPE when engaging in Category 1 tasks.

   Category 3: Routine work does not involve any potential for association with contaminated fluids or tissues nor would they be called upon to do so in an emergency. PPE is not required for the performance of their duties.
2. Universal Precautions
   Due to the potential risks for infection in any given blood, body fluid, or tissue specimen by such agents as the Hepatitis B and C viruses and the HIV virus, it is imperative for all laboratorians to be aware the correct manner in which to process these specimens. Thus, the basic tenant of Universal Precautions is that,

   All blood, body fluid, and tissue specimens must be considered contaminated with an infectious agent.

Such a global view of infection potential has led to the adoption of more stringent measures when handling and processing specimens:

- No smoking in the laboratory
- No eating or drinking in the laboratory
- No storing of food in the laboratory
- No mouth pipetting
- No application of cosmetics
- Use of PPE (gloves, lab coats/gowns, goggles, face shields, etc.)
- Remove of PPE's when leaving the laboratory
- Wash hands with soap and water prior to leaving laboratory
- All items used within a biohazardous area are presumed contaminated (telephones, keyboards, camera, centrifuge, etc.)
- Place needles, blades, and all other sharp objects in heavy leak-proof boxes
- Discard blood and containers to autoclave or incinerator in separate biohazard trash bins.
- Contain aerosol formation when opening capped tubes, blending, sonication or mixing by using a biologic safety hood (Class I or Class II)
- Keep work area and instruments clean and neat. This can be accomplished by wiping surfaces with 0.5% (1:10 dilution) of sodium hypochlorite (bleach) prepared daily or other suitable antibacterial and virocidal disinfectant.
- Avoid wearing sandals, loose clothing, loose jewelry, neckties, and long hair styles (unless tied back or contained)

3. Portals of entry and prominent infectious agents
   a. *Fecal-oral:* primarily Hepatitis A virus (HAV): rarely occurs in the laboratory and then, usually as a consequence of improper handling of patient material. This infection is even more rare in the histocompatibility laboratory, where the majority of specimens handled are tissue or blood. This infection can be avoided entirely by the use of common sense, universal precautions, and soap and water.
   b. *Needlesticks and other "sharps" exposure:* the greatest exposure risk for viral hepatitis and the Human Immunodeficiency Virus (HIV) in the laboratory today. Needle-sticks, glassware/other sharps cuts, or problems arising during venipunctures account for the vast majority of the total number exposure incidents in any health care institution. And, because of the constant association with whole blood and the isolation of lymphocytes, the histocompatibility laboratory is exceptionally vulnerable.
      (1) Exposure guidelines that are established for one's own institution should be prominently displayed in the Quality Assurance Manual.
      (2) Guidelines should include all local, state, and federal recommendations for prevention, surveillance, and monitoring for adherence with Universal Precautions.
         (a) Surveillance must include all needlesticks, cuts, human bites and any other injury that breaks the integrity of skin or mucous membrane and places the involved employee(s) at risk of infection.
         (b) All incidents involving needlesticks and other sharps must be reported according to each respective institution's guidelines and at least a copy of any report generated during an incident must remain in the laboratory.
         (c) All incident reports must show evidence of Director review and follow-up counseling with the employee.
   c. Most common infective agents associated with blood/body fluid/tissue exposure
      (1) Hepatitis B Virus (HBV) – long incubation hepatitis; classic serum hepatitis
         (a) Portal of entry
            - In the U.S. the major mode of HBV transmission is sexual, both homosexual and heterosexual.
            - The parenteral route (entry into the body by a route other than the gastrointestinal tract) transmission , i.e., by shared needles among intravenous drug abusers and to a lesser extent in needlestick injuries or other exposures of health-care professionals to blood, tissue, or body fluids is just as important.
            - Workers are at risk of HBV infection to the extent they are exposed to blood and other body fluids. Employment without that exposure, even in a hospital, carries no greater risk than that for the general population.
         (b) Infection risk controlled mainly through administering vaccine to all employees that have a Category I or II job description. Adequate, cost-effective tests are available to evaluate post exposure immune status.
         (c) Post exposure treatment
            - *Patient originally using needle cannot be identified:* Baseline serology testing done, the puncture victim treated with immune globulin, vaccine may be administered, and immune status checked after 1 and 6 months.
            - *Needle from known hepatitis carrier:* Baseline serology testing done, several doses of hepatitis B immune globulin are routinely given, and the victim's immune status is checked after 1, 6 and 12 months.

(2) Hepatitis C Virus (HCV) – most prominent human fluid and tissue exposure risk today.
   (a) There is no vaccine available for protection and the currently available tests are costly and require molecular capabilities.
   (b) Treatment: Long term interferon
(3) Human Immunodeficiency Virus (HIV) – A very serious concern to health care workers, such that the increasing risk of AIDS transmitted via HIV demands that all precautions must be taken to prevent sharps types of injuries or abrasion and open wound types of exposure.
   (a) Primary transmission of HIV similar to HBV, although it does not occur with as high a frequency as HBV. Exposure may be from either heterosexual or homosexual contact or as a consequence of mucous membrane or parenteral exposure, including open wound exposure to infected blood or other body fluids.
   (b) Post exposure testing is adequate and of moderate expense.
   (c) Treatment: There is neither vaccine nor any other known cure for infection. Multi-faceted and life-long therapeutic drug intervention is required to maintain infected individuals, with limited success.

d. Universal precautions as it relates to the most common blood borne agents
(1) Even though not all body fluids have been shown to transmit infection, because of the ubiquity of the above agents and the great potential for a sharps exposure to occur, all body fluids and tissues must be regarded as potentially contaminated and infectious.
(2) Both HBV and HIV appear to be incapable of penetrating intact skin, but infection may result from infectious fluids coming into contact with mucous membranes or open wounds (including dermatitis) on the skin.
(3) If a procedure involves the potential for skin contact with blood or mucous membranes, appropriate barriers to skin contact must be worn, e.g., gloves, face shields, etc.
   (a) Investigations of HBV risks associated with dental and other procedures that might produce particulates in air, e.g., centrifugation and dialysis, indicated that the particulates generated were relatively large droplets (spatter), and not true aerosols of suspended particulates that would represent a risk of inhalation exposure.
   (b) If there is the potential for splashes or spatter of blood or fluids, face shields or protective eyewear and surgical masks must be worn.
   (c) Detailed protective measures for health-care workers have been addressed by the CDC and can serve as general guides for the specific groups covered, and for the development of comparable procedures in other working environments. Federal Register/Vol. 52, No. 210/Oct '87.

4. Education and Training
a. As stated above, it is mandatory for an institution involved in the handling, processing, and testing of human clinical material to provide employees with education on the relative risks of infection. Dissemination of this information must be part of the initial training of a new employee and must be provided annually as well. Most institutions do this once a year on a global basis and have a log that is signed and dated by the employee upon finishing the initial or refresher training program. Copies of this log and any other documentation of such global training must be made available to the laboratory.
b. For those situations in which the HLA laboratory is responsible for its own biohazard exposure program, a small manual should be developed for initial training and questions concerning this material should appear on initial competency assessment examinations during the early stages of employment. Thereafter, the manual must be read on an annual basis and a log must be signed and dated and/or appropriate questions asked on the annual competency examination.
c. There are many references available on the subject on the relative risk of infection with human clinical material. The literature cited at the end of this chapter lists a few of the most important ones.
d. Any training program for employees on exposure to biohazards must include the following:
   - The OSHA standard for bloodborne pathogens
   - Epidemiology and symptoms of bloodborne diseases
   - Modes of transmission of bloodborne pathogens
   - Institution's Exposure Control Plan (i.e., points of the plan, lines of responsibility, plan implementation, etc.)
   - Procedures used by facility which might result in blood exposure or exposure to other potential infectious materials
   - Methods at facility used to control exposure to blood or other potentially infectious material
   - Types PPE available at facility and where located
   - Personnel to be contacted when potentially infectious blood/tissue/fluid exposure occurs.
   - Post exposure evaluation and follow-up
   - Signs and labels used at facility for potentially infectious processes or materials
   - Facility's Hepatitis B vaccine program

B. Chemical Hazards

Another part of the *"Right to Know Act,"* requires all employers to provide their employees in depth information as to the number, types, and characteristics of all chemicals that they will encounter within the scope of their job description. Additionally, all employers whose personnel are exposed to chemicals in the work place must meet the Hazard Communication Standard (HCS). In laboratories, however, a chemical hygiene plan (CHP) may be implemented which supplants the HCS. This program must have documented evidence of continuous review and oversight by an individual, the chemical hygiene officer (CHO). The CHO may be a member of the department (technologist, supervisor, director) or may operate for the entire institution. The latter is usually a member of the physical plant staff or the safety committee but, in any case, his/her name must be known to all employees.

The Federal Government realizes that each specific laboratory environment is unique. Health care laboratories vary considerably from industry, from other institutions, and even from similar laboratories. Therefore, each facility has been given the autonomy to establish and publish their own program for the use and disposition of chemicals and reagents. These local standards must, in turn, reflect the various regulatory agencies' mandate to protect employees from exposure to hazardous material and must be accessible to each and must be adhered to once implemented. Finally, there must be documented review of the CHP's implementation and the level of adherence by employees.

The Occupational Safety and Health Administration (OSHA), which oversees and ensures employee safety, has inspectors who can and will perform unannounced inspections. These inspectors measure a laboratory's compliance with their institutional plan and they have the power to levy huge fines and, in some instances, close laboratories.

ASHI inspectors also evaluate a laboratory's facilities, environment, and safety. This includes monitoring the laboratory's compliance with their own CHP. If adherence to the plan is marginal or employee training is inadequate or the environment relative to chemical hazards is unsafe to workers or may compromise patient care, the laboratory can have its accreditation revoked. Moreover, because of its deemed status with other regulatory agencies (HCFA, UNOS, JCAHO, OSHA), ASHI is compelled to notify those agencies when such incidences occur. The end result is that the laboratory may have an unannounced follow up inspection by one or more of these agencies and its activities may be severely limited or may even be closed until any deficiencies are rectified.

Because of the individual nature of CHP's, it is necessary that an institution's CHP must reflect their actual practice and not simply parrot some other plan. Blind copying of other plans will leave the laboratory open to potentially severe penalties if it does not abide by its plan, train employees to live by that plan, and monitor that they do live by that plan. Consequently, the CHP should begin with an institutional statement of philosophy. Such a statement should acknowledge the need to implement and maintain a CHP in compliance with the rules and regulations of OSHA, the Environmental Protection Agency (EPA), and state and local governments. The goals of the program are to institute, promote, and maintain a safe working environment that minimizes accidents, reduces the risk of contamination of the environment, and reduces the exposure risk of employees and visitors alike to chemical hazards.

This philosophical statement must also acknowledge the implementation of educational programs to help employees achieve these goals and to ensure proper handling of hazardous chemicals. All employees involved in developing and instituting the plan must be identified, including supervisors responsible for implementing the program, individuals on the committee responsible for developing the plan, and the head of the department whom is legally responsible for ensuring compliance.

1. Essential features of a CHP
   - All hazardous chemicals must be identified.
   - The risk of contamination of employees by hazardous chemicals (by inhalation, ingestion, or skin contact) should be reduced to a minimum.
   - Laboratory employees and employees who handle the waste streams from the laboratory are to be protected.
   - Where appropriate, exposure to these hazards must be monitored to prove that regulatory standards have been met.
   - Medical surveillance is required to limit injury in the event of employee contamination.
   - All hazardous chemicals must be prevented from contaminating the environment
   - Compliance is regulated by the EPA.
2. Hazard Determination

   NOTE: *All hazards in the department must be identified.*

   Many laboratories interpret this as meaning that a list of all hazardous chemicals must be maintained. Another approach is to maintain a list of all chemicals, reagents, and kits used or stored in the laboratory, and then identify all hazardous substances within that list. The master list may be stored in a computerized database, from which lists for individual laboratory sections may be produced.

   a. Material safety data sheets (MSDS): MSDS are required from each manufacturer of chemicals, reagents, and kits and provide the main source of information regarding chemical hazards. They are the simplest and most complete way to accumulate chemical safety data and may be kept in an organized file or notebook or even scanned into a computer (some companies even provide their MSDS on CD Roms). These files or CD-Roms provide readily available information (see list below) for training new employees and as a post exposure reference.
      - Name
      - Manufacturer

- Distributor and relevant catalog numbers
- NFPA code
- Permissible exposure limit (PEL)
- Threshold limit value (TLV)
- Chemical abstract number
- Laboratory section(s) where used/stored
- Location of Materials Safety Data Sheet (MSDS) and the date the MSDS was prepared
- Composition if it is a mixture or kit
- Upper and lower explosion limits (UEL and LEL)
- Whether the chemical is a carcinogenic, reproductive, or acute toxin
- Whether the chemical is corrosive, caustic, flammable, or radioactive and the source of this information
- If EPA regulated, under what section of the law it is regulated

(1) These documents should be kept on permanent file and updated periodically. Updates can be obtained by going out on the internet and looking for MSDS specific websites or by contacting specific manufacturer's websites.
(2) Employees must have ready access to them, in order to handle accidents and for questions from inspectors concerning the MSDS for any chemicals that they might be using.
(3) An employee also has the right to refuse to work with chemicals if they have had no training concerning the hazards involved or have not seen an appropriate MSDS.
(4) The Health Section of the MSDS contains relevant information for hazard determination. All chemicals should be regarded as hazardous until proved otherwise. The manufacturer sometimes will send a letter stating that the material they sell is not hazardous; compliance dictates that the laboratory must either have an MSDS or letter on file for each chemical stating its potential for hazard.

c. *All acute toxins, carcinogens, and reproductive toxins must be identified.* It is advisable that these lists be posted in each section. For a chemical to be recognized as a carcinogen or reproductive toxin, animal or human studies must provide evidence of an association between the chemical and development of cancer, injury to reproductive organs, or the occurrence of abortions or fetal abnormalities. This information is also found within the Health Section of the MSDS. Other sources of information are listed in the reference section.

3. Reducing the Risk of Contamination of Employees
This risk must not be underestimated; however, it may be reduced by:
- Establishing engineering controls
- Modifying employee behavior by training and mandating appropriate laboratory behavior
- Placing adequate warnings in the workplace and in procedures
- Medical surveillance and atmospheric monitoring
- Controlling hazardous chemicals in the laboratory – establishing how they should be transported, stored, and used
- Reducing the accidents by controlling spills and the amounts used.

a. *Establishing engineering controls.*
(1) Ventilation in all rooms should be measured at least annually (preferably more often) and should be adjusted to meet OSHA guidelines or the specific recommendations of reagent or instrument manufacturers. For example: copy machines, which produce ozone, are often overlooked. OSHA has established that machines producing ozone also have ventilation requirements. On average, there must be at least 4 volume air changes per hour; ideally, there should be 10.
(2) Chemical hoods and ancillary ventilation systems should be inspected annually. However, the face velocities of each hood should be checked to see that they are operating.
(3) Simple vanometers are adequate to demonstrate and document that the hood is working. These should be read at least daily if the hood is used regularly or before use if the hood is used infrequently.

b. *Modifying employee behavior.* Employee behavior is modified by establishing training programs and mandating compliance with that training.
(1) *Training Programs* – All employees who work with or are exposed to hazardous materials must be trained before working with any hazardous material, new or old, and whenever there is a change in exposure risk due to procedural or other changes. No employee should be made to work with any physical or chemical hazard for which they have not received appropriate training. Educational requirements should be matched to the job title. All employees must receive refresher safety training at least annually. Employees must sign and date log sheets when they attend these sessions and complete a quiz during each session. The sign-in sheets and quizzes are kept on file for at least three years. Any training plan should encompass the following topics:
- Overall scope of the chemical hygiene plan
- Reading the MSDS
- Selection and use of safety gear
- Spills-how to neutralize, clean up, and dispose of the waste material whether it is acid, caustic, flammable, or a volatile carcinogen

- Understanding chemical labels
- Specific instructions on the use and disposal of all hazardous material that the employee will use in the laboratory
- Carcinogenesis and Toxicology of Chemicals
- Hoods: chemical and biocontainment

(2) *Mandated Behavior.* While working in any part of the laboratory, employees are required to adhere to the following standard operating procedures (SOPs). Any breaks in the integrity of this behavior must be reported, documented, and reviewed by the management staff and the employee counseled where appropriate.

- Carcinogens, acute toxins, and reproductive toxins must be used in designated areas only.
- While working in these areas, employees must wear appropriate protective gear, which include chemical-resistant gloves, apron, and eye protection such as glasses with protective lens or face mask as minimum standard.
- Before leaving the designated area, employees must wash gloves and decontaminate protective gear (unless they are disposable) and remove the clothing. Hands must always be washed when leaving the designated area. Designated areas must be regarded as contaminated areas.
- Eye protection and gloves must be used whenever any chemical is handled or stored.
- Contact lenses must not be worn when working with hazardous chemicals, particularly organic solvents that dissolve in soft lens plastic and thus may be held in intimate contact with the eye for prolonged periods of time. If the employee has to wear contact lenses, airtight goggles must be worn.
- Chemical-resistant, waterproof aprons must be worn when handling hazardous chemicals, particularly if they are in solution or are liquid.
- No hazardous chemical must be given or loaned to unauthorized personnel or anyone who has not received appropriate safety training within the past year.
- Employees must not smell or taste chemicals.
- Food must not be stored in the laboratory, nor can employees eat, drink, smoke, or perform any other activity that brings the hand to the face. Unconscious contamination of the eyes and mouth and subsequent ingestion are major causes of contamination.
- Horseplay or any activity that might startle another employee and cause an accident must be avoided.

(3) *Placing Adequate Warnings.* All chemical and physical hazards must be identified by clear labeling, including NFPA codes. All procedures involving the use of hazardous chemicals should have appropriate safety warnings prominently displayed within the body of the written procedure, and these warnings must be highlighted (this is a College of American Pathologists [CAP] requirement, as opposed to governmental regulation). All designated chemical storage or areas where used must be clearly identified

(4) *Controlling Hazardous Chemicals*

- No hazardous chemical must be stored above five feet from the floor. Accidents can occur when employees reach above eye level for chemicals.
- Fire codes usually limit the amount of flammable materials that can be left in the laboratory. Usually, 500 ml or less of any one chemical is a good rule of thumb. Be sure to check with local and state regulations concerning minimal amounts of chemicals that can be in the laboratory.
- All stock supplies of chemicals must be stored in vented flame cabinets.
- Flammable materials must never be stored in a refrigerator or freezer unless it is explosion-proof. Many organic solvents have flash points at or below refrigerator temperatures, and electrical controls can spark and cause an explosion. Vapor build-up in the refrigerators and freezers, which are usually airtight, can cause vapor concentrations to exceed lower explosion limits.
- Toxic chemicals or flammable chemicals must not be released or stored in airtight areas such as walk-in incubators or refrigerators because of the possible build-up of toxic levels.
- Each section of the laboratory must maintain an inventory of types and volumes of chemicals stored or used within the area and there should be enough spill-absorbent material available to absorb all of the chemical in the largest container stored.
- Highly toxic chemicals must be kept in an unbreakable secondary container.
- When chemicals are carried by hand, they must be carried in a specialized container or bucket.
- Chemicals must be examined at least annually for replacement or deterioration.
- Vent vacuum pumps must be required for all chemical hoods.

c. *Medical Surveillance and Atmospheric Monitoring*

(1) If an employee is contaminated by chemicals as a result of a spill, contamination of skin, or exposure to levels of a chemical that exceed the action limit *(half the permissible exposure limit),* or the employee develops symptoms of exposure after use of the chemical, the employee must be placed under medical supervision.

(2) Atmospheric monitoring is limited to areas where there is a possibility of exceeding action limits, such as areas where xylene and formaldehyde are routinely used.

(3) All other areas must have the hazard risk assessed. This may require additional monitoring or calculation of exposure under worst-case scenarios with the following factors considered:

- The amount of chemical in use at any given time.
- Is the chemical smell present the majority of the time?
- If entire volume of chemical were spilled on the floor at one time would it exceed the action limit?
- Number of air volume changes/unit time.

d. *Protecting the Environment*
Waste control is mandated in order to avoid contamination of the environment. The EPA has the mandate to control pollution of the environment. Sometimes this agency acts through state organizations, which is the case in Texas (i.e., the Texas Water Commission). The Texas Water Commission (TWC) is concerned with chemicals that are dumped into storm sewers, because these drain directly into surface waters and seep to the aquifers. The TWC also monitors effluent from sewage treatment plants and if hazardous chemicals are discharged from a sewage-treatment plants, owners are fined. Chemicals not metabolized by microorganisms in sewage-treatment plants must not be discharged in the sanitary sewer. The local sewage-treatment facility usually has its own regulation concerning sanitary sewer disposal. The EPA publishes a list of chemicals the agency considers hazardous. This is available to the public and is a worthwhile document to have in the laboratory. Factors to consider when storing:

- Keep only one to two months supply on hand at any one time
- Recycle where possible
- No chemicals must be poured down the sink or otherwise introduced into the local sewer system unless local, state and Federal statutes permit it
- A hazardous waste tag signed by the CHO/Safety Officer and the Department Director must accompany all chemical waste picked up for transport off-site
- Only recognized disposal companies must transport and dispose of EPA-regulated substances

C. Emergency Response
As a quick reference for laboratory accidents, emergency responses are given below to limit injuries until appropriate medical care is available.

Type	Problem	Action
Electrical Safety	Burn	1. Pull the plug or cut off current. 2. Check vital signs (heart, breathing). 3. Cover burn with sterile pad or clean sheet. 4. Keep person warm. 5. Call for medical help.
Fire Safety	Flames on Person	1. Drop and roll on ground or wrap in blanket and roll. 2. Use shower or blanket. 3. Use appropriate fire extinguisher. 4. Call for medical help.
	Thermal Burn	1. Remove person form heat or remove heat from person. 2. Apply cold water to the area. 3. Check breathing. 4. Cover burn with sterile pad or clean sheet. 5. Keep person warm. 6. Call for medical help. 7. *Do not use* oils, sprays, or ointments.
Biohazard	Puncture Wound	1. Allow bleeding. Wash thoroughly with warm water and soap. 2. Report to supervisor and employee health department. Save needle/sample for testing.
	Spills	1. Wash skin with soap and water. Wash work area with appropriate disinfectant.
Chemical Hazard	Burns	1. Follow directions on MSD sheets and/or container label for treating exposure to that chemical. 2. Flush with copious amounts of cold water as per local standards and/or neutralize and flush. 3. Cover burns with sterile pad or sheet. 4. Call for medical help. 5. *Do not use* oils, sprays, or ointments.
	Volatile Liquids	1. Follow directions on MSD sheets and/or container label for treating exposure to that chemical. 2. Call for medical help.

III. Radiation Hazards

Overview
Since the last edition of this laboratory manual, there has been an even more significant decline among laboratories using radioactive material for patient testing. There are three major factors responsible for this decline: 1) The development of non-radioactive methods for detecting antigen – antibody reactions or other immunologic mechanisms at a level hitherto thought only possible with radioactive isotopes, 2) The decline in the use of the mixed lymphocyte culture as a result of increased use of molecular typing to define Class II specificity, and 3) The increasing costs of disposing radioactive wastes. However, for those laboratories that still use radioactive decay as a detection method or have the potential to begin using radioactive isotopes, the following section is intended.

A. General Guidelines

1. The quantities of radioactive materials employed in the histocompatibility laboratory and the resulting radiation levels are generally below what is required to cause immediate radiation induced illness. More concern should be placed on the long-term genetic and somatic effects, which can occur as a result of chronic low-level exposures. Since the exact dose at which genetic damage or cancer induction may occur is unknown, common sense dictates that all exposures should be kept as low as reasonably achievable (ALARA).
2. Either the Federal Government or a state agency controls licensing for all laboratories within the United States and each publishes guidelines for the safe use of radioactive materials. Further guidelines are individually determined by each licensed institution to encompass all of the radioactive protocols it is involved in. Each laboratory performing techniques requiring the use of radioactive materials must be licensed by their institution and be familiar with all governing guidelines for the safe use of such materials prior to purchase.

B. Definitions

Listed below are the definitions of a few words that are necessary in order to understand what is required of an institution in the use and disposition of radioactive materials.

1. **ALARA** – As Low As Reasonably Achievable – a commitment made by each licensed laboratory to keep all exposures as low as reasonably achievable. Federal guidelines require that all licensees determine ALARA levels and strive to achieve these levels at all times (typically one tenth of the annual regulatory limit).
2. **Activity** – The number of nuclear transformations occurring in a given quantity of material per unit time.
3. **Alpha Particle** – A charged particle emitted from the nucleus of an atom having a mass and charge equal in magnitude to those of a helium nucleus.
4. **Avalanche** – The multiplicative process in which a single charged particle accelerated by a strong electric field produces additional charged particles through collision with neutral gas molecules.
5. **Becquerel** – A unit of activity. One becquerel equals one nuclear disintegration per second.
6. **Beta Particle** – Charged particle emitted from the nucleus of an atom, with a mass and charge equal in magnitude to that of the electron.
7. **Bremsstrahlung** – Secondary photon radiation produced by deceleration of charged particles passing through matter.
8. **Curie** – The old unit of activity. One curie equals 3.7×10^{10} nuclear transformations per second.
9. **Electron** – A stable elementary particle. Constituent part of an atom often emitted during nuclear disintegration.
10. **Film Badge** – A pack of photographic film that measures radiation exposure for personnel monitoring. The badge may contain two or three films of differing sensitivity and filters to shield parts of the film from certain types of radiation.
11. **Gamma Ray** – A short wavelength electromagnetic radiation of nuclear origin (range of energy from 10 keV to 9 MeV) emitted from the nucleus.
12. **Half-Life** – The time required for a radioactive isotope to lose 50% of its activity by radioactive decay.
13. **Ionizing** – Ionizing radiation includes gamma rays, alpha rays, beta rays and neutrons (any of which can damage cellular DNA), potentially resulting in cell death, cancer or mutation if dosage is high. Conservative assumption would be that any exposure involves some risk. The severity of exposure depends upon the half-life of the radioactive source, the type of radiation, the penetrating power, the part of the body exposed and the duration of exposure. Precautionary measures with regard to use of ionizing radiation in the Histocompatibility Laboratory seek to prevent either internal or external body exposure to the material being used.
14. **Non-ionizing** – Non-ionizing radiation may include microwaves, lasers, ultraviolet light and ultrasonic sources that primarily put the eyes at risk of exposure.
15. **Radioactive Decay** – Disintegration of the nucleus of an unstable nuclide by spontaneous emission of charged particles and/or photons.
16. **Ring Dosimeter** – A monitoring badge in the form of a ring and usually containing a thermoluminescent dosimeter (TLD) chip in place of the film packet.

C. Detectors

1. *Geiger-Muller Counter*
   The Geiger-Muller Counter or GM counter is a gas-filled detector designed to detect any radiation capable of producing ionization within the tube. The GM conducting shell is filled with a gas which has a very low affinity for electrons (i.e. argon, helium or neon). A fine wire is mounted at the center connected to a positive high voltage

source. Any particle entering the tube capable of ionizing even one molecule will initiate an avalanche of ionizations and discharges in the counter that will result in collection of electrons at the center wire. The resulting charge can be measured. This counter measures all types of radiation but for some low energy emitters a thin window is required to allow penetration through the shell.

2. *Scintillation Counter*
   Scintillation counting is an ideal method for quantitating radioactivity since all forms of radiation released, alpha, beta and gamma, can be detected in very small quantities. A scintillation detector consists in its most basic form of a scintillator, a photomultiplier tube and associated circuits for counting light emissions produced by the scintillator. When a charged beta or gamma particle is released into a scintillator it imparts energy to the atoms in the scintillator, which in turn release light proportional to the energy imparted. The photomultiplier tube produces an electrical impulse when stimulated by light emitted from the scintillator, which is used to plot a spectrum for the radiation measured that distinguishes between isotopes.

D. National Radiation Council (NRC) Guidelines

All aspects concerning the production, transportation, possession, use and disposal of radioactive materials is strictly controlled by Federal, State and local authorities. It is crucial that Federal guidelines be extensively researched prior to obtaining any radioactive materials. State regulations are typically patterned after N.R.C. regulations found in the *Code of Federal Regulations, Title 10, parts 19 and 20 (10 CFR 19-20)*. This volume is available at a reasonable cost from any federal government printing office bookshop.

1. *Licensing*
   a. All laboratories anticipating the use of radioactive materials must obtain a license from the proper authorities.
   b. Different types of licenses exist for different institutions.
      (1) *Broad Scope License:* Used by large institutions for all isotopes which are used on the campus.
         - Lists all isotopes used on the campus
         - Does not detail specific procedures.
         - Controlled by a previously approved radiation safety committee within the institution. This safety committee then controls issuance of sublicenses to the individual laboratories or investigators within the institution.

      (2) *Individual license:* For laboratories that are not under the umbrella of a larger institution
         - Must submit extensive procedures
         - Designated safety officer to intercede with authorities and maintain safe operating conditions.

E. Exposure Limits

The standards for maximum permissible dose allowable for radiation workers is set by the NRC or State authorities. The current maximum exposure levels are as follows:

1. Occupational Exposure Areas *(REMS/Year; NCRP Report No. 39, 1971)*
   a. Whole body, lens of eye, red bone marrow, gonads (5)
   b. Hands and feet (75)
   c. Forearms and ankles (30)
   d. Any other specific organ not mentioned above (15)
   e. Fetus gestation period (0.5)
2. Authorities within specific governing areas or the institutional radiation safety officer may place further monthly or quarterly exposure limits.
3. The NRC and most "Agreement States" now require that each institution develop a program to maintain personnel exposures below "ALARA" limits. These limits are set by each institution. Information on specific ALARA limits can be obtained from the Radiation Safety department of each institution.

F. Required Records

1. A complete record must be kept upon receipt of an isotope until its final disposal.
   a. *Large institutions* – materials are usually received in the radiation safety department where all materials are logged in and tested for leakage upon arrival and some of the records concerning these activities or the entire tracking history of a shipment may be kept in the safety office.
   b. *Smaller institutions* – receive, log, and leak test as delivered to them. Individual laboratories are required to keep complete records of a shipments history.
2. Some of the records required are as follows:
   a. *Receipt* – Upon receipt of radioactive materials, detailed records must be filed including all receiving documents. These records must be organized in a logical manner and available for inspection at all times. Upon inspection, laboratory personnel should be able to quickly determine the exact amounts of each isotope or material that they have on hand.
   b. *Leak Testing* – Each package delivered should be tested for container integrity and possible leakage prior to storage or use. These records are often kept on specialized forms. As in all other records the leak testing records must be available for inspection at all times. In the case of large institutions where materials are received in a central location, records for leak testing may be kept in a central area. Clarification of institutional procedures should be obtained prior to licensing.

c. *Use* – Detailed records of use must be kept. Records of amounts used, employee removing, amounts remaining and disposal procedures should be logged for each use. Each laboratory should be able to trace in detail any material received from receipt to removal from laboratory.

d. *Disposal/Waste* – Most of the waste generated in a histocompatibility laboratory has very low levels of radioactivity. Radioactive waste may be generated as liquid, solid or vial form. The waste for each different nuclide should be stored and disposed of separately and according institutional, state, and Federal guidelines.
   (1) A number of different disposal options are available. The method chosen depends on the half-life of the isotope in question, the quantities generated, the concentration of the isotope in the waste and the space available for storage.
   (2) Waste storage and disposal procedures must be developed with proper authorities upon licensing.
   (3) Examples of disposal options available are as follows:
      (a) *Incineration by institution* – facility and institution must be approved prior to use
         • Effluent must be sufficiently dilute to meet requirements for concentrations found in *10 CFR 20 appendix B, Table II.*
         • Records of each incineration must be maintained.
      (b) *Burial* – waste will be packaged by institution and sent for burial in approved site.
         • As of 1993 each state is required to develop burial sites within state boundaries. Until such sites are developed burial of waste will be limited and quite expensive.
         • All institutions in states where no burial sites have been approved are required to obtain approval for onsite storage for varying periods of time.
      (c) *Decay* – Waste is generally stored for a period of time not less than 10 times the half-life of the isotope in question. The waste must then be surveyed prior to disposal.
      (d) *Sanitary Sewer* – It is permissible to dispose of liquid wastes in the sanitary sewer as long as the concentration of radioactivity is less than that considered safe for an adult to drink or breath. Federal or State guidelines should be consulted to determine permissible levels for the areas in question.

e. *Employee Exposure* – Three principal rules govern radiation safety, Time/Distance/Shielding:
   (1) **Time** – exposure is directly related to the amount of time spent in the vicinity of the isotope (i.e. decrease time by one-half and exposure will decrease by one-half)
   (2) **Distance** – the relationship between distance and exposure from a radioactive source is governed by the inverse square law. As the distance increases by a factor of two the exposure decreases by a factor of four.
   (3) **Shielding** – the type of shielding which is required for protection depends on the type and energy of the radioactive emission. Alpha particles impart their energy very quickly and do not penetrate the skin so no shielding is required. Beta particles are generally intermediate in penetrating ability and can best be blocked by acrylic shields. Gamma particle require heavy shielding such as lead or concrete. However, care should be taken to avoid lead shielding for beta emitters as beta particles will interact with lead to produce Bremsstrahlung radiation.

f. *Personnel Monitors*

   As discussed previously, all laboratories using radioactive materials are required to keep detailed records on personnel exposure. Therefore, it is necessary to obtain reliable personnel monitors for personnel working with isotopes. Two different types of monitors are generally used for this purpose, film badges and thermoluminescent dosimeters,
   (1) Film Badges

      Film badges are the most popular type of personnel monitoring device. This badge consists of photographic film sealed inside a labeled packet. The packet is mounted inside a plastic case wedged between shielding of varying types and thickness to distinguish between various energies. This packaging gives a measure of total body exposure and type of radiation. Although the film badge is sensitive, inexpensive and portable some problems do exist. The film can be sensitive to heat and of course light. It is important that the badge be cared for properly and that the package remain intact and to remember that film is not sensitive to very low energy emitters.
   (2) Thermoluminescent Dosimeters (TLD)

      TLD's can be worn as personnel monitors much like film badges. TLD badges are composed of crystalline substances whose electrons are excited to a higher state upon absorption of radiation. When these substances are heated to high temperatures the electrons return to their normal state. Upon return to their normal state energy is released in the form of light. Lithium Fluoride is commonly used used in TLD's. TLD monitors consist of lithium fluoride (or other appropriate materials) sealed inside a labeled, portable holder that can be worn in the same manner as a film badge. Advantages of the TLD are: 1) less sensitive to heat and can detect a much broader range of energies, 2) it gives a permanent record of personnel exposure and, 3) it can be annealed at very high temperatures and reused. However, that in effect destroys any permanent record of personnel exposure. The one great disadvantage of the TLD badge is that it is more expensive.

g. *Contamination/Decontamination*
Should an accident occur involving contamination to an area, immediate attention should be given to localizing the contamination and removing as many personnel as possible from the area. Specific protocols for accidental contamination should be developed by the radiation safety department of each licensed institution. It is important that prior to using radioactive materials all personnel be trained in the safety rules for their prospective institutions. Some general guidelines are listed below:
- Localize the spill to prevent spread to other areas of the lab. If aerosolization is a possibility remove personnel and seal the area.
- Check all personnel for contamination and isolate any who may be contaminated.
- Call appropriate safety personnel for guidance in decontamination. If contamination is below a certain level the lab personnel may clean the contamination up themselves. Institutional guidelines must be followed at all times.
- Decontaminate and survey to determine safety prior to return of personnel.
- Document the incident and keep on file for possible inspection by authorities.
- Should personnel be contaminated, measures to treat or decontaminate should be taken immediately. If the person requires medical attention they should be treated immediately as if the contamination does not exist. Once stabilized or if personnel do not require medical attention the following series of steps should be undertaken:
  i. Personnel must be surveyed with appropriate instruments to determine contamination.
  ii. Contaminated clothing must be removed, bagged and placed in an appropriately shielded area for decay or disposal.
  iii. Skin contamination – care should be taken to prevent spread to other areas of the body. The contaminated area should be washed extensively with a mild detergent and warm water followed by resurveying.
  iv. The procedure should be repeated as necessary until contamination is removed.
  v. Harsh detergents containing lye or hot water should be avoided. Also scrubbing if used should be gentle to avoid penetration of the skin.
  vi. If contamination cannot be removed, help should be sought from safety personnel knowledgeable in alternate decontamination procedures.
  vii. The incident and all procedures used to decontaminate the area must be documented and available to the laboratory.

h. *Employee Training* – Standard operating procedures on the processing, handling, and use of radioactive material must be written and submitted to the regulatory agencies prior to obtaining a license. It is incumbent upon the Director and Supervisor to ensure that all personnel have read these SOP's, are conversant with them, and are accurately following them in their practice. All competency examinations for employees working with radioactive material should have questions dealing with the proper handling and processing of isotopes as well as managing contamination.

i. *Licensing* (see above, D1.)

j. *Safety Surveys* – Work areas, including bench tops, floors and storage areas should be monitored frequently for removable contamination. The most common method of survey is the "wipe test," in which a known area (typically 100 cm^2 or a 10 x 10 cm square) is wiped with a cotton tipped applicator or swab soaked in detergent. The swab is then counted in a scintillation counter appropriately set for each isotope used in the laboratory. Threshold values, above which an area is considered to be contaminated, are determined by each institution. Any area found to be contaminated should be cleaned and resurveyed. All survey values before and after decontamination must be kept for inspection purposes.

3. General Rules of Conduct for personnel working in a radiation environment:
   a. The radioisotope laboratory must be used only for radioisotope work. Unnecessary materials should not be brought into the laboratory, and unnecessary work must not be done there.
   b. Work must be done rapidly but carefully.
   c. Each bottle, flask, tube, etc., which contains radioactive material must be identified by proper radiation warning labels; including amount remaining in the container.
   d. Care must be taken to avoid splashing, splattering, or spilling radioactive liquids.
   e. Smoking, eating, or drinking in the laboratory prohibited at all times.
   f. The laboratory must be kept clean and orderly at all times.
   g. Pipetting by mouth is prohibited.
   h. Absorbent paper must cover work benches, trays, and other work surfaces where radioactive materials are handled and the possibility of spillage might occur.
   i. Disposable plastic or rubber gloves must be worn while working with radioactive solutions when hand contamination is likely.
   j. When procedures are completed, monitor hands for contamination.
   k. Unshielded bottles, flasks, beakers, and other vessels that contain more than 100 mCi of activity must not be picked up by hand for more than a few seconds. Whenever practical and always when the handling time is long, tongs or forceps must be used.

l. Radioactive materials which emit gamma rays and whose activity exceeds 500 mCi must be kept behind lead shields or inside of lead lined vessels. Normally shipping containers are adequate for low level activity storage.
m. PPE must be worn as needed.

References

FACILITIES AND ENVIRONMENT

1. American Society for Histocompatibility and Immunogenetics (ASHI), January,1998. ASHI Standards for Histocompatibility Testing. Kansas City.
2. Code of Federal Regulations, July 1, 1997. Occupational Health and Safety Administration (OSHA) 1910.1000 to end. U.S. Government Printing Office, Washington.
3. Crowe, D, 1998. Quality Assurance in the HLA Laboratory. Southeastern Organ Procurement Foundation (SEOPF), Richmond.
4. Tenover, F. and McGowan, JE, 1995. Section II. Laboratory Management and Regulatory Issues. In: Murray, PR, et.al., Manual of Clinical Microbiology, 6th ed. ASM Press, Washington.
5. Transfusion Service Quality Assurance Committee, AABB, 1997. A Model Quality System for the Transfusion Service. American Association of Blood Banks (AABB), Bethesda.

EXPOSURE TO BIOHAZARDS

1. Assignment of Exposure categories – Joint Advisory Notice; Department of Labor/Department of Health and Human Services; HBV/HIV Notice. Federal Register 52 (210):91821, October 30, 1987.
2. Hepatitis
   a. Centers for Disease Control: Recommendations for protection against viral hepatitis. Morbidity and Mortality Weekly Report 34:313, 329, June 7, 1985.
   b. Centers for Disease Control: Update on Hepatitis Prevention, Morbidity and Mortality Weekly Report 36:353, June 19, 1987.
   c. Koff RS, 1995. Chapter 92. Hepatitis B and Hepatitis D. In: Gorbach SL, Bartlett JG, Blacklow NR, eds. Infectious Diseases (2nd ed.) p850 – 863, WB Saunders, Philadelphia.
3. Human Immune Deficiency Virus
   a. Center for Disease Control: Recommendations for prevention of HIV Transmission in Health-Care Settings. Morbidity and Mortality Weekly Report. 36:25, 1987.
   b. Human T-Lymphotropic Virus Type III-Lymphadenopathy Associated Virus: Agent Summary Statement. Morbidity and Mortality Weekly Report 35:540, 1986.
   c. Resnick L, Veren K, Salahuddin SZ, Tondreau S: Stability and inactivation of HTLVIII/LAV under clinical and laboratory environments. JAMA 255(14):1887, 1986.
   d. Zenilman JM, 1992. Chapter 128. Prevention of Human Immunodeficiency Virus Transmission. In: Gorbach SL, Bartlett JG, Blacklow NR, eds. *Infectious Diseases* (2nd ed.) p1169 – 1183, WB Saunders, Philadelphia.
4. Waste Management
   a. Grument FC, Macpherson JL, Hoppe PA, Smallwood LA: Summary of the Biosafety Workshop. Transfusion 28:502, 1988.
   b. Strain, BA, 1995. Chapter 7. Laboratory safety and Infectious Waste Management. In: Murray PR, Baron EJ, Pfaller MA, Tenover FC, and Yolken RH, eds. *Manual of Clinical Microbiology*. p. 75 – 85 ASM, Washington.
5. General
   a. CDC-NIH Manual. *Biosafety in Microbiological and Biomedical Laboratories.* US Dept. of Health and Human Services, Public Health Service, Center for Disease Control and National Institutes of Health, US Govt. Printing Office, 1984.
   b. Morbidity and Mortality Weekly Report, August 29, 1986.
   c. Morbidity and Mortality Weekly Report, 38(5-6): 1.
   d. Needle Sticks Take a High Toll, The Draw Sheet. University of Virginia Publications, p 30, 1981.
   e. Rose SL: *Clinical Laboratory Safety.* Chapters two, four, and five. J.B. Lippincott Company, Philadelphia, PA, 1984.
   f. Slobadien M: In: *Laboratory Safety, Theory and Practice.* Chapter three, p 60. Fuscaldo A, Erlick BJ, Hindman B, eds. Academic Press, New York, NY, 1980.
   g. Steere NV: Laboratory *Safety, Theory and Practice*, Chapter one, p 4-56. Fuscaldo AA, Erlick BJ, Hindman B, eds. Academic Press, New York, NY, 1980.

HAZARDOUS CHEMICALS

1. EPA Title III List of Lists, Document No. EPA 560/4-91-011 Section 313. Document Distribution Center, P. 0. Box 12505, Cincinnati, OH 45212.
2. NCCLS General Laboratory Practices and Safety Vol. 6, No. 15, Clinical Laboratory Hazardous Waste.
3. Federal Register Vol 55, No 21. Part 1910 of title 29 of the Code of Federal Regulation (CFR), amendment Jan. 31, 1990.
4. Annual Reports. National Toxicology Program. U.S. Department of Health and Human Services.
5. Gregory M, 1995. Chapter 1b. Microbiology Laboratory Safety. In: Mahon CR and Manuselis G, Jr. eds. *Diagnostic Microbiology* p. 32 – 48. WB Saunders, Philadelphia.

RADIATION HAZARDS

1. Noz ME, Maguire GQ Jr: Radiation Protection in the Radiologic and Health Sciences. Lea and Febiger, Philadelphia, PA, 1979.
2. Shapiro J: Radiation Protection: A Guide for Scientists and Physicians. Harvard University Press, Cambridge, MA., 1972.
3. Sorenson JA, Phelps ME: Physics in Nuclear Medicine. W.B. Saunders Co., Philadelphia, PA, 1987.
4. Radiation Regulations and Protection Procedures. Baylor University Medical Center, Revised 1989.
5. Basic Radiation Protection Criteria. NCRP Report No 39, National Council on Radiation Protection and Measurements, Washington, D.C., 1971.
6. Code of Federal Regulations, Title 10, Parts 0 to 50, Office of Federal Register National Archives and Records Administration, Washington, DC, 1988.

Quality Control Program

Anthony L. Roggero and Deborah O. Crowe

Principle and Purpose

The quality of results produced in any laboratory is only as good as the quality of the reagents used. Therefore, one of the most important functions in the laboratory is the quality control of its reagents. Quality control of reagents assures that they are functioning optimally in all tests performed by the laboratory. A reagent is any chemical or biological product used in a laboratory test. It is of critical importance that reagents are properly labeled, stored, prepared, and handled. Failure to do so may adversely affect testing results, and may also pose a threat to the health and safety of testing personnel. Quality in laboratory testing is also dependent upon the performance of equipment and instruments. Proper calibration, routine preventive maintenance, and troubleshooting must be part of the quality assurance program.

A. Components of QC Program

1. Quality Control measures and thresholds for each test performed
2. Criteria for accepting or rejecting results
3. Participation in Proficiency Testing
4. Reagent QC and tolerance limits
5. Equipment calibration and preventive maintenance
6. Documentation of problems and corrective actions

B. Quality Control Measures for Specific Test Methods

1. Each test procedure must indicate quality control measures taken and how they are used in the interpretation of the results. Procedures written in NCCLS format will usually include a Quality Control heading in which the quality control measures are detailed for ensuring that the test is performing as expected.
2. Tolerance levels must be established and corrective actions documented when controls fall outside the expected range. Worksheets used to document QC results should have the tolerance limits clearly indicated.
3. Patient results are not reported if the quality control for the test procedure exceeds the tolerance limits. There must be written criteria for accepting and rejecting results.

Proficiency Testing

1. In-House Proficiency testing – primarily used for tech-to-tech comparisons
2. External Proficiency Testing – the lab must participate in an external proficiency test for every test that is performed in the laboratory. If no commercial proficiency test is available for a test methodology, the lab should attempt to set up parallel testing with another lab that is doing the test at least every 6 months.
3. Review of Proficiency Testing – The director must review proficiency results upon completion of the testing and prior to mailing the results. The director/ technical supervisor must review the findings of the proficiency testing and document discrepancies with the consensus.
4. Corrective actions must be initiated if a result is found to be unacceptable when compared to the consensus result from other labs. Follow-up actions are important to ensure that the corrective action was effective in solving the problem.

Reagent Quality Control

A. Labeling Reagents – a written policy for labeling reagents is required.

1. All reagents must be clearly labeled for identity and concentration of the reagent.
2. The reagent label must indicate the date the reagent was prepared and/or received in the laboratory and the date it was opened or put in use (PIU).
3. The reagent must be labeled with the lot and batch number, the expiration date, and the initials of the person who prepared or opened the reagent.
4. The storage conditions should be included on the label.
5. A hazard warning label must be affixed to every reagent container indicating the type of hazard the reagent may have (e.g. toxic, etc.).

B. Vendor List

1. Establish approved vendor list to ensure vendor qualifications
2. Identify critical equipment, supplies, and services
3. Contract review
4. Establish systems to inspect, log, and store reagents

C. Inventory Control – to ensure adequate supply

1. A **Reagent List** should be kept by the laboratory (see example number 1). This list should contain the following:
   a. Name/Chemical formula where possible
   b. Hazard Category (check for MSDS)
   c. Manufacture source and Cat. #
   d. Preparation Instructions
   e. Storage Requirements
2. A **Reagent Log/Quality Control Record** must be kept to record information on new batches or lots of reagent. (See Examples 2 and 3)
   The log should contain the:
   a. Name of reagent
   b. Lot number
   c. Date received
   d. Expiration date
   e. Date prepared or put in use
   f. Initials of the person preparing or opening the reagent quality control results
3. All reagents MUST be discarded upon expiration.
4. Mixing of reagents between different lots of commercial kits is usually not permitted since the test was validated at the factory using the combination of reagent lots found in the kit.
5. **Reagent Inventory** should be done on a regular basis to ensure adequate supply. For each reagent, one should establish the optimal order size, the lead time needed for ordering, and the quantity remaining when a new order should be made.

D. Documentation of Reagent Performance

1. Every reagent has minimal standards it must meet before it can be placed into use (e.g. pH, support of cell viability, sterility, etc.).
2. Any new reagent lot must be checked for acceptable performance parameters prior to being placed into use to ensure that it is of the same quality and meets the same standards of performance as the reagent currently in use.
3. Any deviation from acceptable ranges necessitates repeat testing and/or consultation with the laboratory Supervisor and/or Director.

E. Procedures for Reagent QC must be written with detailed instructions. Forms should be available to record and document QC performance. The forms must include the tolerance limits for acceptable performance and documentation of review. The procedures should be found in a Reagent QC section of the SOP or in the Reagent QC manual.

1. **Titration Procedures** – for Complement, AHG, and monoclonal antibodies [ex. Monoclonal Antibodies (CD2/CD20, PE) or FITC-anti-IgG reagent for crossmatch]
2. **Parallel Testing** – for typing reagents, magnetic beads, separation media, etc. The old lot and new lot are both tested with the same sample.
3. **Documentation of Acceptable Performance** and "**Date in Use**" for reagents used as "components" in another reagent. This may include such reagents as magnesium chloride, dNTPs, NaCl, etc. For these reagents, one may simply indicate in separate columns on the Reagent Log/QC sheet the date when the reagent was first used and if the test performance was acceptable (see Example 3)
4. **Protein Supplements** (ex. AB serum or FCS) – requires Cytotoxicity testing prior to use. Most easily done by placing on serum screening test
5. **Negative Control** (Normal Human Serum – NHS) – should be tested for Cytotoxicity. Most easily done by placing on serum screening test.
6. **Positive Control** (Anti-Lymphocyte Sera) – Cytotoxicity testing and titer of positive control should be determined – titer should not be higher than seen commonly with alloantisera.
7. **Ability to Support Cell Growth** – Media, Fetal Calf Serum, etc. is checked for its ability to support cell growth for the length of time required by the procedure for which it will be used (ex. MLC).
8. **Special Notes** on the use of some reagents should be included in the procedure for which they are used.

F. Rejection Criteria for Reagents

1. New lots of reagents that significantly differ from expected test results.
2. New lots of reagents that significantly differ from the parallel or previous control test results.
3. Reagents that alter test results.
4. Sterile reagents that failed sterility check.
5. Expired reagents that failed re-quality control testing.
6. Sterile reagents that have not been opened under sterile conditions (under a biological laminar flow hood).
7. Any contaminated bottles must be discarded.

G. Storage Requirements

Reagent Sera	≤ -20°C (≤ -70°C recommended)
Patient Sera	≤ -20°C (≤ -70°C recommended)
Typing Trays:	≤ -70°C to -80°C
PRA trays	≤-70°C to -80°C required;(-135°C/LN_2 recom.)
Complement	≤ -70°C to -80°C
Cells in DMSO	≤ -70°C to -80°C
Tissue Culture reagents	4°C
Immunomagnetic Beads	4°C
Antibiotics	-20°C

Some reagents require special testing prior to use in order to determine the purity, toxicity, or optimum reactivity (titration assays) of the reagent. Of particular concern to the lymphocytotoxicity assay is complement and anti-human globulin reagent quality control. For DNA typing, the most extensive reagent QC is done with Primers and Probes. For Flow Cytometry, the FITC-conjugated anti-IgG reagent requires the most care when determining the optimal working dilution. Because of the complexity of these reagent checks, a brief protocol for each is given below.

Complement QC

All new lots of complement should be tested in parallel with old lots or with defined cell samples on at least 5 tissue typing trays.

"Checkerboard" testing (using dilutions of the new lot of complement vs. dilutions of known antisera) should be performed to determine the strength and toxicity of any new lots of complement (see example 3 for Complement "Checkerboard" form).

Expiration dates for complement and anti-human globulin should be assigned either one year from the date of quality control completion or use the manufacturer's expiration date – whichever is the longer dating. Expired complement and anti-human globulin can undergo re-quality control testing and upon acceptance have the expiration date extended for one year. Any lot that fails re-quality control testing must be discarded.

A. PROCEDURE: New Lot of Complement Evaluation

1. Choose two well-characterized antisera.
2. Choose three well-characterized cells: two that will give positive reactions with the antisera and one that will give negative reactions.
3. Antisera should be used neat (1:1), 1:2 through 1:16. Dilutions can be made with negative (AB) serum.
4. Each dilution is tested with the complement at different dilutions and also with no complement (Complement control or spontaneous lysis control).
5. Complement should be used neat (1:1), 1:2, 1:4, 1:8 and 1:16. Dilutions can be made with appropriate diluent such as RPMI, barbitol buffer, etc.
6. It is essential that new and old lots of complement be tested simultaneously.
7. Positive and negative controls need to be included with each cell tested.
8. A possible tray layout for setting up this complement evaluation, can be found at the end of this chapter.
9. From this study, the complement lot with the best reactivity is chosen. This new lot of complement then needs to be evaluated for use with the laboratory's different test procedures (NIH, AHG, etc.) as well as with different target cells (PBL, B cell, etc.). The complement is tested in parallel with the different crossmatch techniques and with a DR tray to document that it performs satisfactorily under all conditions for use.
10. Care should be taken not to continually reduce the strength of a new lot of complement chosen. This will lead to poorly defined reactions over time, under previously similar test conditions.

B. Special Notes on Complement

1. Complement is heat labile. Long-term storage of complement must be at -65°C or colder.
2. Complement should be kept cold when dispensing aliquots for refreezing. Use an ice bath if aliquoting large quantities.
3. Complement reactivity is destroyed by heating at 56°C for 30 minutes.
4. Gentle mixing when thawing will reduce damage to complement proteins.
5. Violent mixing can cause premature activation.
6. Chelating agents, such as EDTA, can deplete calcium ions necessary for the activation of complement, causing false negative reactivity.

Anti-Human Globulin (AHG) QC

Similar to Complement QC, all new lots of anti-human globulin (AHG) should be tested in parallel with old lots or with defined cell samples.

A strongly positive serum, a serum that reacts with a specified antigen and, if possible, a weak serum that reacts only in the presence of AHG should be used in a "Checkerboard" testing similar to Complement QC (using dilutions of the new lot of AHG vs. dilutions of known antisera). Titers are compared to determine the strength and toxicity for any new lots of AHG.

The AHG titration must include defined cells with and without the antigen for which the serum has specificity. (see example 4 for Anti-Human Globulin "Checkerboard" form).

A. Procedure for AHG Evaluation

1. Choose several well-characterized complement-dependent antisera for testing. These should include a strongly positive serum that reacts with a specified antigen and, if possible, a weak serum that reacts only in the presence of AHG.
2. Choose well-characterized target cells that will react with the antisera selected above.
3. Take a 72 well microtiter tray and dispense 1 µl of the dilutions of one antisera across the tray. Column A on the tray (12 wells) will contain the antisera neat (1:1). Column B will contain the antisera at 1:2, etc.. Column F will contain the negative control.
4. Add 1 µl of a chosen cell preparation to the entire tray. Incubate 30 minutes at room temperature.
5. Wash the tray 3X.
6. Add dilutions of antiglobulin reagent (make reagent and dilutions just prior to use; keep all dilutions cool, 2-6ºC), from the weakest dilution (bottom of tray) to the strongest dilution (top of tray). One dilution is dispensed across an entire row of wells. Row 12 will have a dilution of 1:180 of the antiglobulin dispensed into it and Row 4 will have a dilution of 1:20. Rows 1-3 should not have any antiglobulin reagent dispensed into it.
7. The antiglobulin reagent should only be allowed to sit in the wells for 1-2 minutes prior to adding 5 µl of complement to each well.
8. Incubate the trays an additional 60 minutes at room temperature.
9. Stain cells and record reactions.
10. A possible tray layout for setting up and recording this anti-human globulin reagent evaluation can be found at the end of this Chapter.
11. The optimal dilution of antiglobulin reagent is that which gives 90-100% cell death with the highest dilution of antisera, and highest dilution of antiglobulin reagent. There may be two or three wells (or dilutions) of reagent that demonstrate this maximum efficiency.
12. The optimal dilution of antiglobulin reagent for any cell/serum combination should give at least a two-fold increase in titer strength above that titer observed with the NIH method. Example: If the NIH method gives an "8" (80%+ cell death) at a dilution of antisera of 1:2, the antiglobulin reagent (one or more dilutions) should demonstrate an "8" with a titer at least of 1:8 or greater.
13. Combining the results seen with the different cell/serum combinations, it is possible to choose a dilution of the antiglobulin reagent that will work satisfactorily with most cell/serum combinations.
14. Choose an AHG reagent that has an optimal working dilution of at least 1:16. One that works at 1:64 to 1:256 will allow the laboratory to conserve reagent and preclude the necessity of frequently having to evaluate antiglobulin reagent.
15. Dispense small aliquots of reagent and store at -70°C. Pull a tube, thaw and dilute (with RPMI) the reagent to the appropriate working dilution just prior to use.
    Note: If the AHG reagent is to be used pre-mixed with the complement, the titration should be done in a similar manner. The range of titers used should be approximately 6X that used in the above to account for the "final" concentration of AHG used in the test (1 µl working dilution of AHG + 5 µl of Complement).
    Example: When AHG is titered as described above, start with a 1:20 and go to 1:180. If pre-mixed with Complement, the dilutions tested should include 1:120 to 1:1080 in its range.

B. Monthly Complement and AHG Quality Control

1. On a microtiter tray, dispense a negative control (AB serum) in duplicate.
2. Add a known antiserum in dilutions from neat (1:1) through 1:64 (or higher, depending on titer of antiserum). The same control should be used each month. Dispense the serum dilutions in duplicate. Multiple QC trays may be made and stored at -70ºC for future use.
3. Add a previously prepared cell prep to the quality control tray. The cell chosen must contain the antigen for which the antiserum is specific.
4. Perform the test using the NIH and AHG procedures.
5. Record the titer strength of reactivity. This will be the highest dilution of serum that gives a "6" or "8" reaction.
6. A reduction in titer over time indicates that a new lot of complement needs to be put in use.
7. The titer with the AHG method should be at least 2 dilutions greater than that seen with the NIH method.

Primer QC for DNA Typing

A. New Primer Set QC for SSP Methods

1. Positive Reference DNA Panel
   A panel of reference DNA can be constructed in a set of tubes that parallels the SSP panel to be tested. For example, if the first tube in the SSP panel is specific for DRB1*01, then the first tube in the reference DNA panel contains DNA that has the DRB1*01 gene. Each tube in the DNA panel will be positive with the corresponding tube of the SSP template. The reference DNA may be from a homozygous typing cell (HTC) known to have the allele of the primer mix being tested. Other reference DNA may come from heterozygous individual having the allele

of the primer mix being tested. This can be from a patient that has been previously typed or from a proficiency test sample. The positive panels should show a specific band of the correct size for every well.
Construction of Reference DNA panel:

a. Identify DNA that can be used in the reference panel.
b. Divide 2 by the DNA concentration in µg/µl to determine the amount of DNA to dilute to 100 µl with *complete PCR buffer**. This will give a final concentration of 20 µg/µl.
   * For 50 ml of Complete PCR Buffer
   13.0 ml 10 X PCR Buffer
   923 µl dNTP mix (25mM)
   13.0 ml 25 mM $MgCl_2$
   23.1 ml ddH_2O
c. Place the diluted DNA/PCR buffer mixture in a Reference template that corresponds to the panel being tested.
d. Store in refrigerator or aliquot in smaller amounts and freeze.

2. The SSP panels should contain 5 µl of the appropriate primer mixes in each tube
3. Add 5 µl of the Reference DNA/PCR buffer from the Reference template into the SSP reaction tray. A multichannel pipette may be used for large panels.
4. Prepare a mix of water/Taq polymerase/ 60% sucrose or glycerol according to the following formula:

   n = number of tubes in template + 3

ddwater	n x 1.7 µl
60% sucrose or glycerol	n x 1.3 µl
Taq polymerase	n x 0.05

   Mix and add 3 µl to each tube of reaction tray.

   Total volume = 13 µl. Run the PCR program as usual for the SSP test.

   NOTE: The volumes indicated above may need to be modified slightly if using a commercial kit that requires different volumes. It is important to add about 70-100 ng of reference DNA per tube and then follow the same procedure that that is recommended for the kit being used.
5. Negative Control
   The SSP panel is tested with two or more cells that do not react with the same mixes to show that the primers are specific. Only control bands should be present in the negative tubes. If a specificity problem is suspected, or if a primer mix has been known to be troublesome in the past, the primer mix should be tested with a known Reference DNA that is very close to the specificity of the primer mix to ensure specificity (i.e. run allele 0402 against 0403 primer mix to show specificity with a closely related allele).
6. Complete Typing of Reference DNA
   In addition, a single Reference DNA may be run with a full set of primers (complete typing). The value of a full typing is that one can more effectively evaluate the presence of nonspecific bands and/or cross-reactive products. In addition, the presence of all the expected bands for a known type can be assured. This is especially valuable when designing a new panel or primer mix or when a problem arises which requires that the specificity of a primer mix be verified.
7. When performing quality control on a reagent, all other reagents used in the procedure must have been previously tested and found satisfactory.
8. It is also a good idea to repeat the QC in parallel with the next lot to document the stability of the reagents during storage and as a comparison with the new lot. Once the storage conditions have been validated, the end-of run parallel testing does not have to be continued unless the storage conditions are changed.

B. Monitoring of Primer Mix Reactivity

1. All aberrant results observed during the use of a lot of primer mixes should be recorded.
2. Continuous review of these reactions is necessary to determine the cause for the discrepancies (ex. cross-hybridization with similar sequence on another allele). Knowledge of aberrant reactions is vital when interpreting results.
3. The identification of new reaction patterns should be documented.

Probe QC for DNA Typing

A. SSOP Probe Labeling QC

1. After labeling, each probe is tested with reference DNA to ensure sensitivity and specificity of the reaction.
2. The results are recorded on the probe QC worksheet.
3. It is recommended that a panel of reference DNA be included with each SSOP run.

B. Reverse SSOP

1. For reverse SSOP developed in-house, new lots must be validated with sufficient reference DNA to ensure proper reactivity with each probe.

2. For commercial DNA typing kits, a reference DNA should be run prior to use with patient samples. Additional reference DNA should be tested periodically to monitor performance of the probes. The reference DNA should be rotated so that in the course of the year, most of the probes have been tested.

C. SSOP and Reverse SSOP Primer QC

1. After PCR, the PCR product is run on gel electrophoresis to determine if amplified product of the appropriate size is obtained. No further testing is done (Dot blot or ELISA) if no product is observed.
2. If no product is observed, one must troubleshoot to determine if the problem lies in the DNA sample or with one of the components of the PCR mix.

Titration of FITC-anti-human IgG for Flow Crossmatching

A. Goat Anti-human IgG

1. Anti-IgG heavy chain or Fc-specific reagent coupled with FITC
2. $F(ab')_2$ fragments have lower background binding
3. Specificity important – should not react with other Ig classes; affinity purified – pre-absorb with other Ig classes coupled to solid phase support

B. Titration of Anti-human IgG

1. Usually purchased in 1 mg vial. Reconstitute with 0.75 ml of H_2O and 0.75 ml of glycerol. Store in 10 µl aliquots in freezer
2. Concentration of reconstituted anti-IgG = 1 mg/1.5 ml = 0.67 mg/ml = 0.67 µg/µl
3. For titer, one should cover a range from 0.2 µg to 1 µg per test.
   The optimal amount is also dependent on the final volume of test.
   For example, one may wish to add 20 µl of the working dilution of the FITC-anti IgG to each test. If 20 µl contains 1 µg, then 1 µl would contain 0.05 µg.

   Dilution needed to make 0.05 µg/µl: 0.67 mg/ml ÷ 0.05 = 14.5

   Make 1:14.5 dilution of stock by adding 135 µl PBS to 10 µl stock = 0.05 µg/µl
4. The working dilution made above is then further diluted (ex. 1:2, 1:3, 1:4) and 20 µl of each dilution is used per tube in the crossmatch test. This should give a range from 0.25 to 1.0 µg per test when 20 µl of each working dilution is used.
5. Examine data and choose which working dilution gives optimal result. The dilution to use in future tests will be 14.5 x the secondary dilution used. For example, if the 1:3 dilution of the original 1:14.5 dilution gave the optimal results, then 3 x 14.5 or a working dilution of 1:43.5 should be made (10 µl aliquot + 425 µl PBS)
6. Document results of titer in Reagent QC record. Compare performance of new lot with old lot.

Equipment Maintenance

1. Written protocols for Preventive maintenance
2. Written schedule for maintenance checks – incorporate required frequency of maintenance checks
3. Documentation of maintenance checks – results recorded and stored in Maintenance Manual
4. Tolerance limits set for each maintenance check. The tolerance limits should appear on the worksheet on which the results are recorded.
5. Corrective actions and follow-up when results are outside tolerance limits.
   a. Written procedure for troubleshooting problem
   b. Written procedure for repairing instrument (if applicable)
   c. Back-up procedure or instrument
   d. Notification of proper persons with details of malfunction
   e. Back-up plan in case of power failure

References

1. ASHI Laboratory Manual, 3rd Edition, 1994. Section VI.6 Quality Control.
2. Standards, ASHI, 1996.
3. CAP Inspection Checklist, 1996.
4. ASHI Accreditation Standards Guidelines and Checklist, March 15, 1995.
5. DCI Laboratory Procedure Manual, Nashville, TN 1998

Example 1

REAGENT PRODUCT INFORMATION

Name	Manufacturer	Health Hazard	Preparation	Storage Req.	
				Temp	Shelf Life
Sodium Citrate, $Na_3C_6H_5O_7.2H_2O$	Sigma	skin irritant	stock chemical	22° C	indef.
PBS / 0.6% Citrate	Fisher	skin irritant	6 g NaCitrate dissolved in 1 liter PBS	0-8° C	6 mo.
EDTA, $CH_{10}H_{14}N_2O_8Na_2.2H_2O$	Fisher	skin, eye irritant	stock chemical	22° C	indef.
PBS/8% EDTA	Fisher	skin, eye irritant	40 g Disodium EDTA dissolved in 500 ml PBS. pH to 7.4 with NaOH.	2-8° C	6 mo.
Sodium Hydroxide, NaOH	Fisher	caustic	30 g NaOH dissolved in 100 ml deionized water	22° C	1 year

Example 2

REAGENT / MEDIA LOG and QUALITY CONTROL

_____ PBS _____ PBS/0.6% Citrate
_____ McCoy's Media _____ McCoy's with AB serum
_____ Fluoroquench _____ LSM
_____ Dynal Beads I / II _____ Other _____

Manufacturer	
Lot Number	
Previous Lot Number	
Received / Prepared Date	
Expiration Date	
Date Placed into use	

Quality Control Checks:

1. **Lymphocyte Processing**
The percentage of cell viability of a cell preparation using the new reagent is a reflection of its performance.

Tolerance Limit: Viability should be > 90%
Results: % Cell Viability = _____ Pass / Fail

Tech: _____ Date: _____

2. **Cytotoxicity Assay**
Processing reagents or media utilized in the lymphocytotoxicity test must show a score of "1" for the Negative control (AB serum) and a score of "8" with the positive control (ALS). Results are recorded for six consecutive tests.

Results:

Negative Control						
Positive Control						

Tech: _____ Date: _____ Pass / Fail

3. **pH** = _____ (PBS/0.6% Citrate = pH 7.0 – 7.2) Pass / Fail
(McCoy's Media = pH 6.6 – 7.1)

Tech: _____ Date: _____

Reviewed by: _____ Date: _____

Example 3

MISCELLANEOUS REAGENT QC

Year:_____

Reagent	Lot (Date Made)	Date Tested	Sample Tested	Pass/Fail	Tech	Review

COMPLEMENT TITER

Manufacturer: _____ Date tested: _____

Lot # : _____ Exp. date:_____ Tech: _____

Antiserum spec. _____ ID:_____

Cell phenotype: _____

			Complement Dilutions					
			A	**B**	**C**	**D**	**E**	**F**
	Serum Dilution		**Neat**	**1:2**	**1:4**	**1:8**	**1:16**	**Normal Serum No C′**
Neat	1	C′ Control; Buffer instead of serum						
Neat	2	Neg Control						
Neat	3	Antiserum						
1:2	4	"						
1:4	5	"						
1:8	6	"						
1:16	7	"						
Neat	8	Pos Control						
Neat	9	B cell Control						
	10							
	11							
	12							

Results:
C′ titer = _____

Comparable to old lot? Yes / No Acceptable? Yes / No

Reviewed by: _____ Date: _____

ANTIGLOBULIN TITER

Manufacturer: _____ Date tested: _____

Lot # : _____ Exp. date:_____ Tech: _____

Antiserum spec. _____ ID:_____

Cell phenotype: _____

			AHG Dilutions					
			A	**B**	**C**	**D**	**E**	**F**
	Serum Dilution		**Neat**	**1:2**	**1:4**	**1:8**	**1:16**	**B cell Control**
Neat	1	Pos Control						
Neat	2	Neg Control						
	3	Antiserum, no AHG						
1:20	4	"						
1:40	5	"						
1:60	6	"						
1:80	7	"						
1:100	8	"						
1:120	9	"						
1:140	10	"						
1:160	11	"						
1:180	12	"						

Optimal AHG dilution = _____

Comparable to old lot? Yes / No Acceptable? Yes / No

Reviewed by: _____ Date: _____

Synthesis of Rare DRB1 Alleles for SSOP

Debra D. Hiraki, Shalini Krishnaswamy and Carl F. Grumet

Principle and Purpose

Validation of any HLA typing method should demonstrate that the test is capable of reacting properly with all alleles it claims to be able to identify. For example, if the assay is based on oligonucleotide probes (SSOP) recognizing short stretches (~20 bp) of DNA within a PCR product, the assay is best validated if the probe can be tested against all the sequence variations known to exist within the target sequence of the probe. Generally, differences outside of the probe site are not as important to probe reactivity as those within the probe site. Further, some probes may not hybridize as predicted, and therefore probe reactivity should always be verified under actual test conditions. Oligonucleotides are sometimes substituted as test targets in SSOP validations; however their reactivity may differ substantially from that of the whole amplicon, necessitating the use of real PCR amplicons. Since many alleles currently recognized are very rare, finding test DNAs to use for validation of all alleles may be difficult or impossible.

An alternative to finding the rare alleles is to simply synthesize them. Rare alleles are generally very close in sequence to some common alleles, differing in only one or two bases. End differences, i.e. those present within 30 base pairs of a primer site can be incorporated into the synthetic product simply by using a newly designed, extended primer in a second round of PCR amplification (We have used this technique to generate DRB1*1316, *1328 and *0423 amplicons). For middle differences, i.e. those more internal than 30 base pairs, the similarity to common alleles can be exploited by using the technique of overlap PCR. This technique utilizes two internal primers to introduce the rare allele's DNA sequence into it's closest common allele to yield a product similar (or identical) to that used in the assay. Figures 1 and 2 illustrate overlap PCR applied to the synthesis of a DRB1*1426 product. (We have also used this method to generate DRB1*0703 and *1506 amplicons.) Synthesis of rare DR alleles is thus feasible and offers the best available test material for complete validation of molecular typing methods.

Specimen

The initial amplicon for this procedure needs to be a single DRB1 allele closest in sequence to the desired rare allele (i.e. differing in only 1 or 2 closely positioned base pairs within the entire amplicon.) The starting genomic DNA chosen to produce the initial amplicon therefore must be of an HLA type that not only possesses the desired closely related allele, but also is either homozygous for DRB1 or possesses a second allele that will not amplify with the chosen primers. Furthermore, the primers should be chosen so that there will be no amplification of DRB3, 4 or 5 locus products. For example, when the rare DRB1*1426 was sought, the GH46-CRX37 primer pair could be used with any DR2, DR1401 heterozygote since that primer pair amplifies only DRB1 products, but not DR2, 7 and 9 alleles.

Figure 1. Genomic and primer sequences for the creation of DRB1*1426
This figure shows the relevant genomic sequence for DRB1*1426 and the closely related, more common allele DRB1*1401. The biotinylated sense and antisense primers were designed to introduce into a 1401 amplicon the new A in codon 24 of 1426 using overlap PCR.

RELEVANT GENOMIC SEQUENCES:

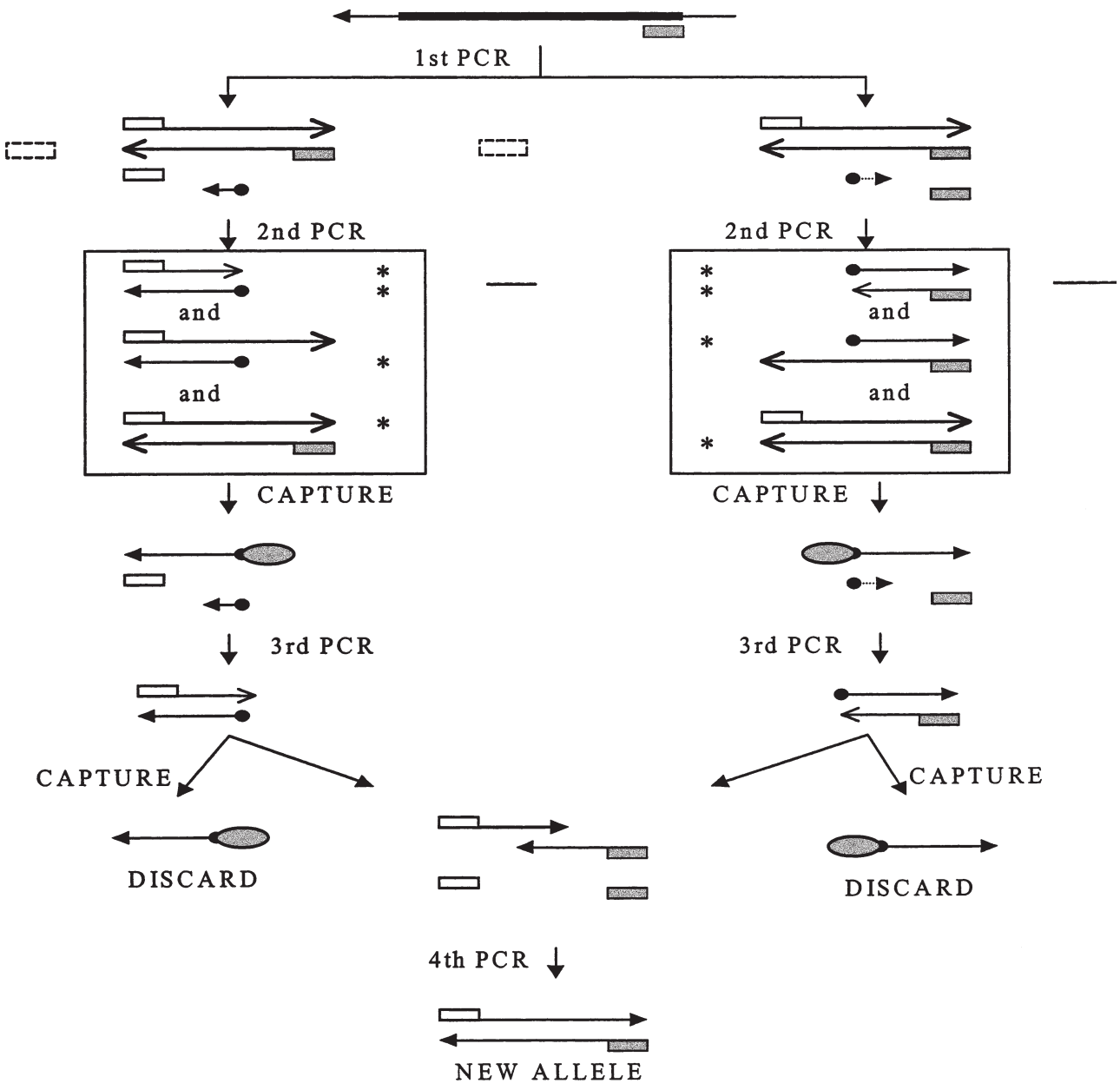
DRB1*1426: ...5′ TGG GAC GGA GCG GGT GC**A** GTT CCT GGA CAG ATA CT...

DRB1*1401: ...5′ TGG GAC GGA GCG GGT GC**T** GTT CCT GGA CAG ATA CT...

PRIMERS:

BIOTIN-DR1426 Sense	5′ Bio-GA GCG GGT GC**A** GTT CCT GGA C 3′
BIOTIN-DR1426 Antisense	5′ Bio-GA GCG GGT GC**T** GTT CCT GGA C 3′
CRX37	5′ GAA TTC CCG CGC CGC GCT 3′

Figure 2. The generation of a synthetic DRB1 allele:



Fuller length, non-mutated fragments persist and are generated in the early steps. These non-mutated fragments could subsequently increase the background of unmodified, original amplicon and interfere with the duplexing of the desired mutated half-strands. To eliminate the contamination, the mutated strands are isolated on streptavidin-coated magnetic beads. The duplex is denatured and all contaminating strands are removed. A third round of amplification yields pure mutated products. After another round of capture and denaturation, the biotinylated strands are discarded and the non-biotinylated strands are allowed to duplex to form the template for the new allele, amplified with the original primers. GH46; CRX37; SA streptavidin-coated magnetic beads; * newly generated strands in this round of PCR; biotinylated antisense DRB1*1426 primer; biotinylated sense DRB1*1426 primer.

Reagents and Supplies

Primers:

1. Redesigned primer for one end extended in the 3′ direction to include the new desired allelic bases
   OR
   Biotinylated 20-mer primers in both the sense and antisense directions, completely overlapping and designed with the desired new allelic base substitution(s) centered in both primers.
2. Streptavidin-coated Dynabeads M-280
3. Standard PCR reagents
   a. TEN (10mM Tris, pH 7.5; 1mM EDTA; 2M NaCl)
   b. 0.1 N NaOH
   c. TE (10mM Tris, pH 7.5; 1 mM EDTA)
   d. 0.8 N HCl
   e. Standard agarose gel

Instrumentation and Special Equipment

Magnet for Dynabead separation
Thermocycler
Agarose gel electrophoresis equipment

Quality Control

Prepare a substantial amount of product for future use and store aliquots at -70° C. Use as reference DNA with quality control of new probe mixtures.

Procedure

FOR ALLELES WITH NEW POLYMORPHIC POSITIONS WITHIN 30bp OF A PRIMER:

1. Redesign the closest primer to extend up to (and, if necessary, past) the sites of the desired introductions, up to 45bp in length. If the final primer is too long, the primer may be then shortened on the 5′ end to make a usable primer. The final product will then be just a few bases shorter than the regular test amplicon.
2. Amplify with your regular primer pair (as discussed under Specimen.)
3. Dilute the product 10^{-5} to 10^{-7} and reamplify with the newly designed primer and the original primer going in the other direction. Verify clean amplification on an agarose gel.

FOR ALLELES WITH NEW POLYMORPHIC POSITIONS MORE THAN 30bp AWAY FROM A PRIMER: (The following steps are diagrammed in Figure 2.)

1. Amplify the chosen genomic DNA with your regular primer pair (as discussed under Specimen.)
2. Dilute the original product 10^{-4} to 10^{-6} and reamplify to give 2 fragments:
   a. Original left hand primer (sense) with the new biotinylated antisense primer to give a left hand product.
   b. Original right hand primer (antisense) with the new biotinylated sense primer to give a right hand product.
   c. These two new products overlap and are complementary on the 3′ terminus of their mutated strands. Verify clean, single band amplification for each on an agarose gel.
3. Isolate biotinylated strands from contaminating whole, non-mutated strands:
   a. Prepare 2 aliquots of 20 µl avidin-coated Dynabeads per manufacturer's instructions.
   b. Resuspend each aliquot of beads in 40 µl TEN and mix one with 40 µl left hand product and the other with 40 µl right hand product.
   c. Bind 15 min, room temperature with rotation or occasional shaking. Wash with 40 µl TEN.
   d. Denature the non-biotinylated strand with 10 µl 0.1 N NaOH for 10 min, room temperature.
   e. Remove the NaOH containing the nonbiotinylated strand.
   f. Wash the beads with 50 µl 0.1N NaOH, followed sequentially by 50 µl TEN, 50 µl TE and final resuspension in 40 µl DDW
4. Amplify only mutated templates: Dilute beaded biotinylated products 10^{-2}. Repeat last pair of amplifications. Verify clean, single-band amplification on an agarose gel.
5. Stitch together the proper fragments: Since now only mutated fragments are present and since the two fragments are complementary, a new template DNA can be generated by allowing the fragments to anneal at their mutated ends, i.e. duplexing the non-biotinylated strands from each reaction.
   a. Prepare 40 µl avidin-Dynabeads as above with resuspension in 80 µl TEN.
   b. Mix **both** products (40 µl each) and beads together and bind 15 min, room temperature.
   c. Wash the beads with 100 µl TEN.
   d. Denature with 20 µl 0.1 N NaOH. **Remove and save the NaOH supernatant with the nonbiotinylated strands to a new tube.**
   e. Neutralize immediately with 3 µl 0.8 N HCl.
   f. Dilute with an additional 50 µl water or 10 mM Tris, pH 7.5.

6. This mixture does not store long. Amplify immediately at 10^{-1} to 10^{-4} dilution of above mixture with original primers (e.g., CRX37 – GH46) to identify the best dilution for amplification. Verify clean amplification on an agarose gel. Amplify a large quantity of product for use and storage.

Results

The new product should now contain the desired allele sequence. Verify by sequencing. Use this new product in the validation of any assay required. Because this product will be very pure, be sure to use a suitable dilution in your validation assays.

Procedure Notes

1. If the products at any stage are not single bands for some reason, it may be necessary to run the product on an agarose gel, cut out the desired band and purify it on a spin column before proceeding with the Dynabeads and subsequent amplification.
2. Although this procedure was used to synthesize oligonucleotides that can be used for an SSOP method, it may possible to use this product with SSP assays as well. However, in order to prevent cross-hybridization and false positive results, one must optimize the dilution of the synthesized product. In addition, the synthesized oligo should be mixed with DNA from a cell containing a similar allele in a proportion that would represent its normal frequency in a DNA extract.

Limitations of Procedure

1. Failure to find a starting DNA of a type which will allow the single, unique amplification of one desired DRB1 allele or the use of primers which amplify anything in addition to the one DRB1 allele will result in a mixture of products and inaccurate validation.
2. Titration of the synthesized product is necessary to determine the optimal dilution for best sensitivity and specificity.

References

1. Horton RM, Hunt HD, Ho SN, Pullen JK and Pease LR, Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. Gene 77: 61-68, 1989.
2. Behar, E., Lin, X., Grumet, F.C., Mignot, E. A new DRB1*1202 allele (DRB1*12022) found in association with DQA1*0102 and DQB1*0602 in two Black narcoleptic subjects. Immunogenetics 41:52, 1995.

Quality Control for DNA Contamination

Jeffrey M. McCormack

Principle

The polymerase chain reaction is a very powerful tool that can be used to amplify segments of DNA a million-fold or more. One of the dangers of using this technique is contamination of the laboratory with amplicons which can be re-amplified in subsequent PCR runs. An important part of quality assurance in laboratories performing PCR is to monitor for DNA contamination. DNA contamination, either genomic or amplicon, could conceivably yield false positive results, and as a consequence, erroneous reporting. Therefore, strict criteria have been established for molecular typing laboratories to perform routine tests aimed at identifying DNA contamination.[3]

Acceptable means for controlling DNA contamination include the use of ultraviolet (UV) irradiation,[7,8] uracil-DNA glycosylase,[9-11] hydroxylamine hydrochloride[12] and exonuclease III.[13] While these methods are in most cases adequate, it is still important to have a reliable method to monitor the effectiveness of de-contamination efforts and to identify potential problems with contaminating DNA or amplicons. Laboratories performing molecular histocompatibility typing are required to monitor DNA contamination by regular wipe tests, testing negative controls (no DNA), open tubes, etc.[3] The purpose of the wipe test is to survey laboratory surfaces and equipment for DNA contaminants and then take appropriate steps to decontaminate areas which test positive. Similarly, the use of open tube controls and negative controls provide a means to monitor for aerosolized DNA and contaminated reagents, respectively.

Appropriate objectives to effectively monitor contamination include 1) the design of an oligonucleotide primer set specific for nonpolymorphic regions of class I and/or II for use as a control primer set; 2) establish and validate a PCR-based wipe test procedure and 3) verify the use of the primer set for detecting PCR products generated by the method being used.

To monitor for Class II amplicons, a primer set, RBQBf/RBQBr was developed which is specific for nonpolymorphic regions of the DR-, DQ- and DP- consensus sequences. The expected PCR products are 81 bp (DR- and DP-) and 79 bp (DQ-). RBQBf/RBQBr detects genomic DNA from reference cell lines LWAGS and BM21 (50-100 picograms) as well as DR-, DP- and DQ- amplicon (1 copy). Additionally, RBQBf/RBQBr detects SSP-PCR products from clinical DR- and DQ-class II typings.

Validation studies employing controlled DNA contamination of laboratory surfaces revealed that increasing amounts of wipe test sample (5-20%) were inhibitory to the wipe test PCR, whereas lower amounts (1-2%) or, alternatively, a diluted wipe test sample, increased the sensitivity of the test and optimized the results. It was also observed that inhibitory factors introduced into the PCR during the wipe test process may yield false negative results. The Wipe Test must be designed to have optimal sensitivity and the validity of negative results must be confirmed by testing for inhibitory factors. This is routinely done by spiking a second PCR test with a known amount of DNA amplicons.

Materials and Reagents

Wipe Test Primers

RBQBf	GCT TCG ACA GCG ACG TG
RBQBr	CCT TCT GGC TGT TCC AGT ACT C

Wipe test PCR mix
1x PCR buffer
500 µM deoxynucleotide triphosphates (dNTP's)
2.5 mM $MgCl_2$, 1x PCR buffer
0.5 µM of each oligonucleotide primer
Taq polymerase
Agarose gel
4% agarose gel made with 3:1 Nusieve Agarose (FMC Bioproducts, Rockland ME) or 2% agarose
Positive Control –
a pre-amplified PCR product is serially diluted from 1:1000 to 1:1,000,000 and tested with the wipe test PCR mix. The product should be detected up to at least 1:10,000. Choose the highest dilution that gives a strong positive band as the working dilution to use for the positive control in the Wipe test.
Filter paper or a Puritan cotton-tipped applicator
(3 in.; Hardwood Products Company, Guilford, ME) for wiping test areas
Forceps
Purified, nitrocellulose-filtered water
Isopropanol

PCR Cycling Conditions
95° C, 30 seconds
60° C, 15 seconds
72° C, 15 seconds for 30 cycles

Procedure

Wipe tests should be taken from the DNA isolation area, the PCR set-up area, the clean room bench area, the floor of the clean room, the reagent preparation area, the thermal cyclers, and the electrophoresis area.

Each wipe test sample is amplified with the designated "wipe test primers" that are capable of detecting all PCR products as well as genomic DNA contamination.

The internal control primers are also included in the wipe test primer mix. A duplicate PCR test is set up which is spiked with DNA or a dilution of PCR product. This is run to ensure that there is not extraneous matter in the wipe test sample that is interfering with or inhibiting the Taq polymerase. Score "+" or "-" for presence or absence of a PCR product on the gel.

A. Wiping Procedure

1. Decontaminate forceps isopropanol and rinse in ultrapure water or use sterile disposable forceps.
2. Wet 1.5 cm diameter disk of filter paper in ultrapure water using the forceps.
3. Wipe filter paper or swab over a 10 cm square area.
4. Place filter paper or swab in a 1.5 ml microfuge tube with 120 µl ultrapure water and vortex.
5. Incubate at 56° C for 1 hour. Centrifuge at 7000 rpm for 30 seconds. Store in refrigerator until tested.

B. PCR for Wipe Test

1. Aliquot 8 µl Wipe Test PCR mix into 16 PCR tubes. Also add the Wipe test PCR mix to a tube that has been opened on the work area for at least one day (Open tube control).
   NOTE: It is suggested that a batch of Wipe test PCR mix be made and pre-aliquotted into strips of PCR tubes. These can be stored frozen until needed. The mix will need to be added to the Open Tube control on the day of testing.
2. Arrange the tubes for one Positive, one Neg (No DNA), one test sample for each area wiped, one spiked sample for each area wiped, and one Open tube Negative.
3. Add 2 µl of supernatant from each wipe test sample to the appropriate duplicate tubes.
4. In a separate tube, mix 40 µl sucrose or glycerol with 1 µl Taq polymerase. Add 2 µl to each of the tubes.
5. Add 2 µl of known positive sample to the Positive control tube and to one of the duplicate wipe test samples.
6. Amplify the wipe test samples and controls using the lab's standard amplification protocol.
7. PCR products can be electrophoresis on a 4% agarose gel made with 3:1 Nusieve Agarose or a 2% agarose and subsequently visualized and documented by ethidium-bromide staining, UV transillumination and photography.

Results

The results are recorded on a worksheet.

Area Wiped	PCR Result	Spiked PCR Result
DNA Isolation Hood		
PCR Set-up Hood		
Reagent Preparation Area		
DNA Clean Room Floor		
Thermal Cycler Area		
Fume Hood / Gel Preparation Area		
Post-amplification Bench Area		

Positive Control _____ Open Tube Negative Control_____ Pass / Fail

Contaminated Areas

Contaminated areas should be cleaned thoroughly with 1M HCl or 10% bleach. Wipe tests should be repeated and should be negative (with exception of possibly the post-amplification areas) before work continues.

Contaminated Area	Date Cleaned	Retest Results

Interpretation

1. There should be a PCR product present in the Positive control tube. No product should be present in the Negative control.
2. There should be a PCR product in the "spiked" tubes for each of the wipe test areas. The absence of a PCR product in these tube suggests that the reaction may have been inhibited by materials present in the wipe test sample. If the spiked sample fails to show a product, the corresponding "unspiked" wipe test cannot be interpreted.
3. The presence of a PCR product in the unspiked wipe tests indicates contamination with genomic DNA or amplicons. De-contamination procedures should in instituted immediately and the wipe test repeated to verify that the contaminants have been successfully removed.

Quality Control

1. A Positive control is included with each run. The positive control can be genomic DNA (25 ng/µl) or a dilution of PCR product to test the ability of the primers to detect contamination.
2. A Negative Control and/or Open tube negative control is included with each run. The negative control contains no known source of DNA and is used to identify contamination in reagents used in the test or from aerosols (open tube control).
3. Spiked controls are set up with each of the test samples. A duplicate of the test sample is spiked with a known amount of positive control. Failure of the spiked sample to amplify suggests that there may have been something picked up from the wipe test that is inhibiting the reaction. For example, bleach residue has been known to inhibit the polymerase reaction and thus invalidate the test.

Validation Procedures

Introduction

When the RBQBf and RBQBr primers were first designed, it was necessary to validate their ability to detect low amounts of DNA contamination, both genomic and amplicon. The following describes the procedures that were undertaken to validate this test. It is not necessary for each laboratory to repeat this validation if using the same wipe test primer set. However, if additional primers are needed (for example, to detect Class I amplicons), a similar approach may be taken.

RBQBf/RBQBr Primer Design

An optimal set of primers for use in monitoring DNA contamination would identify genomic DNA as well as amplified PCR products generated from the polymorphic region of exon 2 of the HLA class II B genes. Examination of the polymorphic and nonpolymorphic regions of exon 2 revealed areas which would serve as likely primer annealing sites and meet these criteria. The polymorphic regions include: DR- (amino acid positions 9-13, 25-38 and 67-74), DQ- (amino acid positions 26-37, 52-57, 70-74) and DP- (amino acid positions 8-11, 33-36, 55-57, 65-69, 72-76 and 84-87). The nonpolymorphic regions nested between areas of polymorphism were selected as potential targets for wipe test oligonucleotide class II primer annealing sites. Using PRIMER ((Version 0.5 by Stephen E. Lincoln, Mark J. Daly and Eric S. Lander, MIT Center for Genome Research and Whitehead Institute for Biomedical Research), a software package for designing oligonucleotide primers, RBQBf/RBQBr primer set was selected. The forward primer (RBQBf) anneals at nucleotides encoding amino acids 39-44 for DR- and DQ-, and 37-42 for DP-. The reverse primer (RBQBr) anneals at nucleotides encoding amino acids 59-66 for DR- and DQ-, and 57-64 for DP-.

WQLKF/G86r primer set are modified oligonucleotides previously reported[9] and amplify DRB1*0101/0103 alleles yielding a 257 bp product.

DPAMP-A/DPAMP-B primer set generates a 327 bp product and are generic primers which amplify all DPB1 alleles.

QB1D/GILQRR primer set are modified oligonucleotides previously reported[10] and amplify DQB1*05/06 resulting in a 268 bp product.

All PCR primers were synthesized using Applied Biosystems 380B and 381A DNA Synthesizers.

Control DNA

Homozygous typing cells used for characterizing RBQBf/RBQBr primers were:

KOSE (A2, B35, Cw12, DRB1*1302/1401, DQB1*05031/0604),
WT100BIS (A11, B35, Cw4, DRB1*0101, DQB1*0501), and
BM21 (A1, B41, Cw17, DRB1*1101, DQB1*0301).

Genomic DNA from patients samples were isolated using Genomix (Washington Biotechnology, Bethesda MD) per the manufacturers instructions. Genomic DNA from homozygous typing cells was isolated by phenol/chloroform extraction.

DR-, DQ- and DP- Amplification and Purification

1. DR-, DQ- and DP- amplicons were generated using various oligonucleotide primers previously reported.[10]
2. The forward primer (RBQBf) anneals at nucleotides encoding amino acids 39-44 for DR- and DQ-, and 37-42 for DP-. The reverse primer (RBQBr) anneals at nucleotides encoding amino acids 59-66 for DR- and DQ-, and 57-64 for DP-. RBQBf/RBQBr generates 81 bp PCR products from DR- and DQ-, and DP-.
3. PCR conditions were identical to those described for RBQBf/RBQBr with the exception of the cycling conditions. PCR cycling conditions were:
   1) DR-, 95° C, 30 seconds; 60° C, 15 seconds; 72° C, 15 seconds for 30 cycles
   2) DP- 95° C, 30 seconds; 70° C, 30 seconds for 30 cycles
   3) DQ- 95° C, 20 seconds; 55° C, 50 seconds; 73° C, 20 seconds for 34 cycles.
4. The purification of PCR products was accomplished by excising the desired bands from the agarose gel following electrophoresis and then using Wizard PCR Preps DNA Purification System (Promega Corp., Madison, WI).

SSP-PCR Typing

1. SSP-PCR typing was performed using the UCLA PCR-Amplification Mixtures (UCLA Tissue Typing Laboratory, Los Angeles, CA). as described by the manufacturer.
2. The PCR was performed using a Perkin-Elmer 9600 thermal cycler for 32 cycles using the following cycling parameters: 95° C, 15 seconds; 58° C, 15 seconds; 73° C, 10 seconds.
3. PCR products were analyzed by electrophoresis and photographed as described above. For analysis of PCR products, generated bands were excised from the agarose gel, melted and used as template DNA for RBQBf/RBQBr amplification.

Validation Test

1. In order to validate the established wipe test procedure, known amounts of genomic and amplified DNA were used to contaminate laboratory surfaces.
2. Ten-fold serial dilutions (250 µl) of genomic DNA or amplicon were applied to previously decontaminated laboratory bench surfaces and allowed to dry overnight.
3. The samples were applied to defined benchtop areas to ensure that DNA would be acquired during the validation procedure. The samples were recovered and processed as described above in the wipe test procedure.
4. Experiments assessing the presence of inhibitory factors in wipe test samples were accomplished by mixing varying amounts of a wipe test sample with known amounts of amplified DNA.
5. In each case the PCR product was quantitated using NIH Image, a public domain program designed for digital image processing and analysis.
6. The integrated intensity of each band was determined and the percent inhibition was calculated using as 0% inhibition a PCR reaction without wipe test sample.

Results

The expected PCR product generated from DR-, DP- and DQ- class II genes is 81 bp. The PCR products generated using primer sets WQLKF/G86r, DPAMP-A/DPAMP-B and QB1D/GILQRR will result in products which include the non-polymorphic regions recognized by RBQBf/RBQBr primer set, therefore making these PCR products useful tool for evaluating the effectiveness of RBQBf/RBQBr in detecting DR-, DQ-, DP- and amplicons.

A. Detection of Genomic and Amplified DNA Using RBQBf/RBQBr Primer Set

The sensitivity of the primer set RBQBf/RBQBr was first determined by testing serial dilutions of target genomic DNA from reference cell lines LWAGS and BM21. Using two-fold serial dilutions of genomic DNA, it was determined that the primer set was capable of detecting between 50-100 picograms of genomic DNA. Likewise, purified DRB1*0101 amplicon was quantitated and used as target DNA and RBQBf/RBQBr was able to detect a single copy of purified DRB1*0101 PCR product. Similar results were obtained using purified DP- and DQ- amplicon, thus demonstrating that the primer set RBQBf/RBQBr is capable of detecting low levels of both genomic (50-100 picograms) and amplified DNA (single copy).

B. RBQBf/RBQBr Detection of DR-, DP- and DQ- Amplicon

Using target DNA from reference cell lines WT100BIS and KOSE and a patient sample, PCR products were generated for DRB1*0101 DQB1*05031/0604, DP- 1 respectively, using primer sets previously described. Amplicon were purified as described in Materials and Methods and used as target DNA to assess whether RBQBf/RBQBr primer set could detect amplicon generated from the class II genes DR-, DQ-, and DP-. PCR products generated using RBQBf/RBQBr to detect amplicon clearly showed that RBQBf/RBQBr satisfactorily detects all three amplicon. These data demonstrate that RBQBf/RBQBr will serve as a mechanism for detecting PCR products generated from all class II genes.

C. Inhibition of Amplicon Detection with Increasing Wipe Test Sample Volume

In order to verify the effectiveness of the RBQBf/RBQBr primer set, a validation process was established which consisted of controlled contamination of laboratory surfaces and subsequent detection of the contamination using the wipe test procedure. However, a significant observation made in the initial phase of the validation protocol was that when using published procedures calling for 20% of the PCR test to be wipe test sample,[3] false negative results were consistently observed from areas known to be contaminated. One approach to explaining the observed false negative

results was to determine whether inhibitory factors from the wipe test samples were being introduced into the PCR-based test. To test this hypothesis, varying amounts of a routine wipe test sample (2-20% final PCR volume) was added to known amounts of amplicon to determine if the test samples would inhibit the PCR. When using 20,000 copies of DRB1*0101 amplicon as target DNA, and 20%, 15% or 10% of the PCR volume consisting of wipe test sample, 100% inhibition of the PCR was observed. Inhibition of 90% was observed using 5% sample and 48% inhibition when 2% of the final volume was the wipe test sample. These data clearly demonstrate that significant amplicon contamination (20,000 copies) may yield false negative results when wipe test samples are added at increasing amounts (5-20%). Moreover, it is possible that lower levels of DNA contamination might go undetected using wipe test samples equal to or less than 1-2% of the PCR. For example, a single amplicon contaminating a surface might go undetected due to inhibitory factors with the addition of less than 1% of wipe test sample.

D. Detection of SSP-PCR Typing Amplicon

The primer set RBQBf/RBQBr was able to detect low levels of both genomic and amplified DNA. However, the definitive test to assess the value of RBQBf/RBQBr as tools to monitor DNA contamination in the molecular typing lab was to determine the effectiveness in detecting PCR products generated in routine laboratory typings. To accomplish this, random SSP-PCR products were sampled from an SSP-PCR typing methodology, the UCLA PCR-Amplification Mixtures from the UCLA Tissue Typing Laboratory, Los Angeles, CA. The results of sampling PCR products generated from a clinical typing and then using the amplified PCR product as target DNA for RBQBf/RBQBr. Samples which were selected indicated that the PCR results when the samples were used as targets for RBQBf/RBQBr amplification. Clearly all PCR products generated from the typing served as a suitable template for RBQBf/RBQBr amplification. Taken together these results showed that RBQBf/RBQBr is an efficient primer set for detecting amplicon generated from SSP-PCR histocompatibility typing.

Discussion

The level of polymorphism of the human major histocompatibility complex (HLA) has historically been a major obstacle to generating thorough histocompatibility testing. Recently however, PCR-based approaches have exploited the genetic intricacy of the HLA complex in developing molecular typing methods which produce, in many cases, definitive results. While the results are indeed favorable, the use of PCR methods introduces a new set of QC issues relating to the increased sensitivity inherent to the PCR. It is imperative that laboratories adhere to strict guidelines regarding protective clothing, laboratory design and workflow to minimize potential DNA contamination. Moreover, laboratories are required to monitor DNA contamination by weekly wipe tests, utilization of open tube controls during DNA isolation and testing negative controls (no DNA) samples. Compliance with these regulations demands close scrutiny of the design, validation and implementation of QC procedures used in monitoring DNA.

References

1. Hurley, C, Yang SY: Quality assurance and quality control for amplification-based typing. ASHI Laboratory Manual, 1995, V1.13.1.
2. Ou, CY, Moore, JL, Schochetman G: Use of UV irradiation to reduce false positivity in the polymerase chain reaction. Biotechniques 10:442, 1991.
3. Pang J, Modlin J, Yolken R: Use of modified nucleotides and uracil-DNA glycosylase (UNG) for the control of contamination in the PCR-based amplification of RNA. Mol Cell Probes 6:251, 1992.
4. Thornton CG, Hartley JL, Rashtchian A: Utilizing uracil DNA glycosylase to control carryover contamination in PCR: characterization of residual UDG activity following thermal cycling. Biotechniques 13:180, 1992.
5. Longo MC, Berneinger MS, Hartley JL: Use of uracil DNA glycosylase to control carry-over contamination in the polymerase chain reaction. Gene 93:125, 1990.
6. Aslanzadeh J: Application of hydroxylamine hydrochloride for post-PCR sterilization. Mol Cell Probes 7:145, 1993.
7. Zhu YS, Isaacs ST, Cimino G, Hearst JE: The use of exonuclease III for polymerase chain reaction sterilization. Nucleic Acids Res 19:2511, 1993.
8. Sarkar G, Sommer SS: Parameters affecting susceptibility of PCR contamination to UV inactivation. Biotechniques 10:590, 1991.
9. Olerup O, Zetterquist H: HLA-DR typing by PCR amplification with sequence-specific primers (SSP-PCR) in 2 hours: an alternative to serological DR typing in clinical practice including donor-recipient matching in cadaveric transplantation. Tissue Antigens 39:2257, 1992.
10. McCormack, JM, Sherman M, Mauer DH. Quality control for DNA contamination in laboratories using PCR-based class II HLA typing methods. Human Immunology 54 (1):82, 1997.

The Joint Commission on Accreditation of Healthcare Organizations

Anne Belanger

The Joint Commission evaluates and accredits nearly 20,000 health care organizations and programs in the United States. An independent, not-for-profit, Self-supporting organization, the Joint Commission is the nation's predominant standards setting and accrediting body in health care. Since 1951, the Joint Commission has developed state-of-the-art, professionally based standards and -valuated the compliance of health care organizations against these benchmarks. Joint Commission evaluation and accreditation services are provided for a wide-variety of health care organizations including hospitals, home care organizations, nursing homes, and many types of clinical laboratories.

The Joint Commission's corporate members are the -American College of Physician American Society of Internal Medicine, the American College of Surgeons, the American Dental Association, the American Hospital Association, and the American Medical Association. Governance consists of a 28-member Board of Commissioners including nurses, physicians, consumers, administrators, providers, employers, labor representatives, health plan leaders, quality experts, ethicists, health insurance administrators and educators. The board brings to the Joint Commission countless years of diverse experience in health care, business and public policy.

The Joint Commission accredits approximately 2,700 organizations that provide laboratory services., including independent laboratories and laboratories in other types of accredited health care organizations. Laboratories eligible for accreditation include:

- Laboratories in hospitals, clinics, long term care Facilities, home care organizations, behavioral health organizations, research labs, ambulatory sites and physician offices;
- Independent laboratories performing specialty testing of all types as well as routine testing-Blood transfusion and donor centers;
- Governmental laboratories, such as Indian Health Service, Veterans Administration and military outpatient laboratories.

The Joint Commission uses performance-focused standards that emphasize the results a laboratory should achieve, rather than specific methods of compliance. The standards manual contains many examples of how compliance might be achieved in various types of laboratory settings for each standard. Laboratories may follow examples as written, modify the examples to suit their own situation, or develop their own path to compliance. As long as the laboratory meets the intent of the standard, compliance is assured.

In 1995, the Joint Commission launched a cooperative accreditation initiative to reduce redundancy and overlap in the accreditation of health care organizations. The initiative focused on improving the efficiency, and reducing the cost of quality oversight activities by enhancing the communication and coordination among various public and private sector organizations that have responsibility for these activities. This initiative, cemented by written agreements, permits the Joint Commission to substantially rely on the process, findings, and decisions of other accrediting bodies in circumstances where the Joint Commission would otherwise conduct potentially duplicative surveys of organizations seeking accreditation.

Under these cooperative agreements, the Joint Commission will accept the accreditation decision of the other accrediting body or government agency for specific components of health care organizations undergoing Joint Commission review. For those Joint Commission standard areas not covered by the other accrediting body, the Joint Commission may conduct a limited survey.

Organizations with cooperative agreements have passed an extensive review of their standards and standards development process; survey process; selection, training and monitoring of surveyors; and accreditation decision process. They have also agreed to maintain an approach to public disclosure, comparable to the Joint Commission's approach. Beside the American Society for Histocompatibility, and Immunogenetics, the Joint Commission has also finalized cooperative accreditation agreements with seven other professional organizations with accreditation including American Association for Ambulatory Health Care (AAAHC), American College of Radiology Radiation Oncology Program, CARF, The Rehabilitation Accreditation Commission (Medical Rehabilitation Program), and the College of American Pathologists. The cooperative agreements with ASHI, CAP, CARF Medical Rehabilitation, COLA and CHAP apply to all accreditation programs. The cooperative agreements with AAAHC, ACR Radiation Oncology and CoC apply only to the Network Accreditation Program and will be reevaluated at a later date for applicability to other accreditation programs.

In addition, the Joint Commission has interimagreements with six other organizations which apply only to the Network accreditation Program. These interim agreements are currently being evaluated for potential future cooperative agreements.

For more information about the Joint Commission and all its accreditation programs, educational products and services, consumers and the health care community can access the web site at www.jcaho.org.

ASHI – The HCFA Perspective

Sandra Pearson and Esther-Marie Carmichael

What is DHHS?

The **DEPARTMENT OF HEALTH AND HUMAN SERVICES (DHHS)** is the government's principal agency for protecting the health of all Americans and providing essential services, especially for those who are least able to help themselves. The DHHS includes more than 300 programs, covering a wide spectrum of activities, such as, medical and social science research; infectious disease prevention (immunizations); assuring food and drug safety; Medicare and Medicaid health insurance programs; financial assistance for low-income families; child support enforcement; improving maternal and infant health, head start, preventing child abuse and domestic violence, substance abuse treatment and prevention, services for older Americans, comprehensive health services delivery for American Indians and Alaska Natives. The Office of the Secretary provides leadership.

Divisions under DHHS include:

National Institutes of Health
Centers for Disease Control & Prevention
Indian Health Service
Substance Abuse & Mental Health Services Administration
Health Care Financing Administration
Administration on Aging
Food and Drug Administration
Agency for Toxic Substances and Disease Registry
Health Resources & Services Administration
Agency for Health Care Policy and Research
Administration for Children and Families

What is HCFA?

The **HEALTH CARE FINANCING ADMINISTRATION (HCFA)** is the federal agency that administers the Medicare, Medicaid, and Child Health Insurance Programs. HCFA helps pay the medical bills for more than 75 million beneficiaries. HCFA also regulates all laboratory testing (except for research). Approximately 158,000 laboratory entities fall within HCFA's regulatory responsibility. HCFA's responsibilities include:

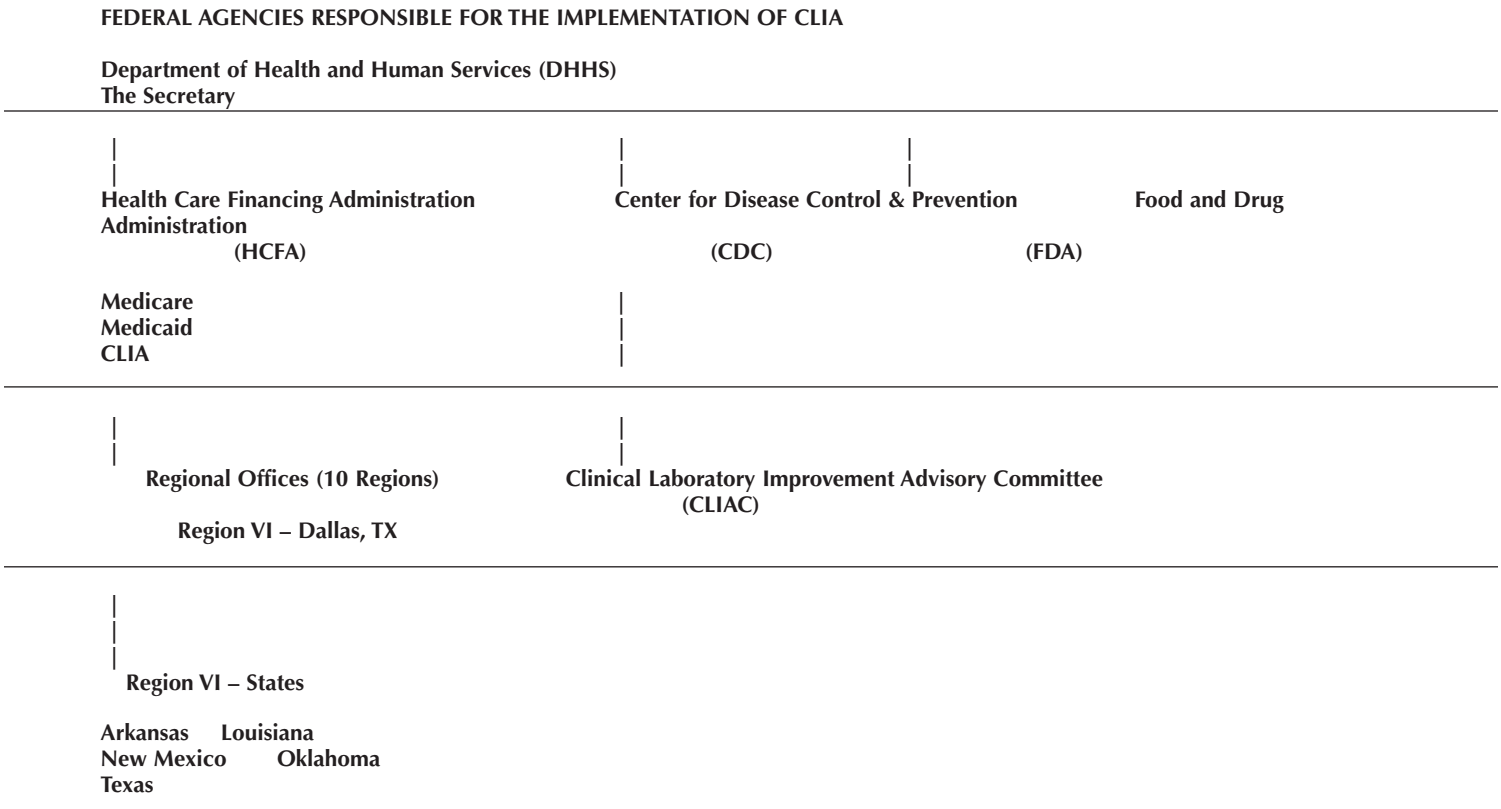
- assurance that the Medicaid, Medicare, and Children's Health Insurance programs are properly run by its contractors and state agencies;
- establishes policies for paying health care providers;
- conducts research on the effectiveness of various methods of health care management, treatment, and financing;
- assess the quality of health care facilities and services and taking enforcement actions as appropriate;
- areas of special focus:
  fighting fraud and abuse; and
  improving the quality of health care provided to the beneficiaries by:
  – developing and enforcing standards through surveillance;
  – measuring and improving outcomes of care;
  – educating health care providers about quality improvement opportunities; and
  – public education to encourage good health care choices.

HCFA's structure includes their headquarters located in Baltimore, Maryland, with 10 Regional Offices nationwide overseeing the HCFA programs. The headquarters staff are responsible for national program direction and national reporting. The Regional Office staff provides HCFA with the local presence necessary for quality customer protection and service and program oversight. The Regional Office locations are available on the Internet at www.hcfa.gov/medicaid/clia/cliahome.htm.

CLIA Authority

CLIA is the Clinical Laboratory Improvement Amendments of 1988. The responsibility for carrying out CLIA is vested in the Secretary of Health and Human Services (HHS) under Section 353 of the Public Health Service Act, as amended. The new section 353 required the Department of HHS to establish certification requirements for any laboratory that performs tests on human specimens, and certify through issuance of a certificate that those laboratories meet the certificate requirements established by HHS.

The Secretary of HHS then delegated to HCFA the responsibility for the implementation of CLIA, including laboratory registration, fee collection, surveys, surveyor guidelines and training, enforcement, approval of Proficiency Testing (PT) providers, accrediting organizations and exempt states. The Centers for Disease Control and Prevention (CDC) has been responsible for test categorization, development of technical standards, and CLIA studies. Within HCFA, the Division of Outcomes and Improvements, within the Family and Children's Health Program Group, under the Center for Medicaid and State Operations (within HCFA) has the responsibility for implementing the CLIA program.



Summary of Agency Responsibilities Under CLIA

Federal Agencies With Responsibilities Under CLIA

In order for FDA, CDC and HCFA to carry out the different functions of CLIA, the agencies entered into interagency agreements allocating responsibilities and funding.

HCFA Responsibilities:

- Approve and monitor Proficiency Testing programs;
- Approve and monitor accrediting organizations for deemed status;
- Approve and monitor State programs; granting their laboratories exemption from CLIA;
- Develop administrative regulatory requirements;
- Implement new and revised regulatory requirements;
- Develop guidelines and survey process for surveyors;
- Develop and support a data system for information collection, analysis and reporting requirements;
- Collect fees, enroll and certify laboratories;
- Survey laboratories, ensure compliance, and take enforcement action;
- Develop, implement, and monitor the CLIA budget;
- Design and conduct training sessions for CLIA surveyors; and
- Ensure fiscal solvency of the CLIA Program which is the only fee-funded government program

CDC Responsibilities:

- Measure effectiveness of CLIA through analysis and research;
- Manage the Clinical Laboratory Improvement Advisory Committee (CLIAC);
- Develop and revise technical regulatory requirements;
- Collaborate with HCFA to:
  - Develop standards, policies and guidelines
  - Evaluate State programs for approval
  - Evaluate accrediting organizations for deemed status
  - Monitor and evaluate PT programs

FDA Responsibilities:

- Categorize tests;
- Approve tests for waiver, PPMP;

NOTE: *Under the CLIA regulations issued in 1992, the FDA was to categorize new commercial test systems as part of the 510(k) and PMA approval process. Due to financial and resource constraints, the FDA was unable to implement these tasks in the early 1990s. CDC was to categorize all in-use test systems and non-commercial test systems on request from anufacturers, users, or developers of the test system.*

Manufacturers and Congress have expressed concern that having both the CDC and FDA participate in product reviews creates "confusion, and duplication of effort". Currently, HHS is working with CDC and FDA in transitioning the responsibility for test categorization to FDA.

What is CLIA-88?

CLIA is the Clinical Laboratory Improvement Amendments of 1988. Congress passed CLIA-88, as a means for the Secretary of Health to develop comprehensive, quality standards for all laboratory testing to ensure the accuracy, reliability and timeliness of patient test results regardless of where the test was performed. A laboratory is defined as any facility which performs laboratory testing on specimens derived from humans for the purpose of providing information for the diagnosis, prevention, treatment of disease, or impairment of, or assessment of health. CLIA is a user fee funded government program; therefore, all costs of administering the program must be covered by the regulated facilities. Facilities that do not accept Medicare or Medicaid or only accept cash, or provide free laboratory testing must be certified under CLIA. It is the act of performing a laboratory test that defines the requirement of certification and not how the test is paid for. CLIA is payment neutral.

The final CLIA regulations were published on February 28, 1992 and were based on the complexity of the test method; thus, the more complicated the test, the more stringent the requirements. Three categories of tests have been established: waived complexity, moderate complexity, including the subcategory of provider-performed microscopy (PPM), and high complexity. CLIA specifies quality standards for proficiency testing (PT), patient test management, quality control, personnel and quality assurance.

Data indicates that CLIA has improved the quality of testing in the United States. The total number of quality deficiencies has decreased approximately 40% from the first cycle of laboratory surveys to the second cycle of surveys. Current PT review data concurs with these earlier findings. Due to the educational value of PT in laboratories, CLIA-88 continues to address initial PT failures with an educational, rather than punitive, approach.

Background

Prior to CLIA, HCFA regulated laboratories under two federal programs: Medicare/Medicaid and CLIA'67. HCFA had two Memoranda of Understanding (MOUs):

- In 1979 (revised 1987) an MOU agreement was signed between HCFA and the Centers for Disease Control (CDC) for provision of scientific and technical expertise on questions relating to advances in instrumentation, new technology, proficiency testing, and cytology services. In addition, prior to 1979, CDC had the responsibility for the regulation of CLIA-67 licensed laboratories. In 1979, HCFA became responsible for the regulation of these laboratories.
- In 1980, an MOU was signed between HCFA and the FDA (Food and Drug Administration) for the provision of technical assistance concerning blood bank services. HCFA assumed the responsibility for the inspection of Registered Blood Establishments that also participate in Medicare. These include transfusion facilities that were located in accredited hospitals either to collect and/or transfuse whole blood, packed cells, and/or other blood components in emergency situations.

These arrangements are longstanding and are based on department policy to coordinate activities and reduce duplicate inspections.

Legislative History

CLIA-67; Clinical Laboratory Improvement Act of 1967 [P.L. 90-174]:

To implement CLIA-67, section 5(a) Part F of title III of the Public Health Service (PHS) Act (42 U.S.C. 262-3) was amended by the changing the title to read: "Licensing — Biological Products and Clinical Laboratories" and by adding section 353 (42 (U.S.C.) 263). Section 353 regulated any laboratory engaged in interstate commerce, that is, soliciting or accepting (directly or indirectly) any specimen for laboratory examination or other laboratory procedures and required CLIA-67 licensure. Laboratories were given a full, partial, or exempt CLIA-67 license, depending on the scope of laboratory testing. Regulations included Applicability; License – Application & Renewal; Quality Control; Personnel Standards; Proficiency Testing; Accreditation; General Provisions; and Sanctions.

Medicare/Medicaid; Independent and Hospital Laboratories;

Only independent and hospital laboratories seeking Medicare/Medicaid reimbursement were regulated under Title XVIII and Title XIX of the Social Security Act. Each facility type had their own regulations to follow.

Medicare/Medicaid/CLIA-67 Regulations: August 5, 1988- Proposed [March 14, 1990 – final and effective 09/01/90]:

In April 1986, a study [Final Report on Assessment of Clinical laboratory Regulations] on clinical laboratories recommended that HHS review the existing regulations to determine how to improve the assurance of quality laboratory testing and achieve program uniformity.

The August, 1988, proposal sought to recodify the regulations for these programs [Hospital laboratories, Section 1861(e) of the Social Security Act (SSA); Independent laboratories, 1861(s)(11) and 1861(s)(12) and (13); CLIA-67, Section 353 of the Public Health Service (PHS) Act [42 U.S.C. 263(a)] interstate commerce; Medicaid, Section 1902(a)(9)(C) of the SSA] into a new Part 493 in order to simplify administration and unify the health and safety requirements for all programs as much as possible.

CLIA-88:

Beginning in 1987, a series of newspaper and magazine articles were published on the quality of laboratory testing. Also, simultaneously television programs were aired concerning the number of laboratories that were not subject to either federal or state regulations. Congress held hearings in 1988 and heard testimony from "victims"of faulty laboratory testing. Specific concerns were raised about the validity of cholesterol screening and the accuracy of Pap smear results.

Section 4064 of the Omnibus Budget Reconciliation Act of 1987 [OBRA-87 – Public Law 100-203], enacted on December 22, 1987, amended Section 1861(s)(11) to require physician offices that performed more than 5000 tests per year to meet regulations. Laboratory testing in both physicians' offices (POLs) and rural health clinics that did not accept and perform tests on referral specimens would not be subject to these revisions because both the Medicare and CLIA statues [Section 1861(s)(11) of the Act and section 351(I) of the PHS Act] respectively preclude the regulation at this time of POLs and RHC that perform tests only for their own patients.

On October 31, 1988, Congress enacted Public Law 100-578 in response to the congressional hearings. PL 100-578 greatly revised the authority (PHS Act) for the regulation of laboratories.This law revised section 353 of the PHS Act (42 U.S.C. 263a) amending CLIA-67 by expanding the Department of HHS's authority from regulation of laboratories that only accepted and tested specimens in interstate commerce to the regulation of any laboratory that tested specimens for the diagnosis, prevention, or treatment of any disease or impairment of, or the assessment of the health of human beings.

Congress then enacted OBRA-89 (Public Law 101-239) on December 19, 1989. Section 6141 removed the provision under section 4064 of OBRA-87, which would now require certification of all laboratories performing tests. In addition, it required laboratories participating in the Medicare/Medicaid programs to comply with CLIA'88 requirements.

On February 28, 1992, the final regulations for CLIA-88 were published with an implementation date of September 1, 1992. Sections of the CLIA requirements were to be phased in allowing previously non-regulated laboratories to get used to the regulations. The regulations adding Provider-Performed Microscopy Procedures (PPMP) were published on March 24, 1995. Work is currently in progress with the CDC and HCFA to develop final CLIA regulations, which will reflect all comments received since the September 1, 1992, Federal Register publication and the development of new technologies.

CLIA Certificates

To enroll in the CLIA program, laboratories must first register by completing an application, pay their certification and/or compliance fees, and if applicable, undergoes an inspection to become certified. CLIA fees are based on the certificate requested by the laboratory (that is, waived, PPM, accreditation, or compliance) and the annual test volume and types of testing performed. Waived and PPM laboratories may apply directly for their certificate as they aren't subject to routine inspections.

Those laboratories which must be surveyed routinely; i.e., those performing moderate and/or high complexity testing, may choose to meet CLIA requirements through HCFA or their agent (State Survey Agency) or an approved, private accrediting organization. The HCFA survey process is outcome oriented and utilizes a quality assurance focus and an educational approach to assess compliance.

Process Overview

A laboratory must obtain CLIA certification for any onsite laboratory testing. A CLIA application, form HCFA-116 can be obtained from either a HCFA Regional Office or State Survey Agency. Internet address: www.hcfa.gov/medicaid/clia/cliahome.htm.

The laboratory must complete the HCFA-116 (and any other additional information/forms that the State Survey Agency or Regional Office requests) and return the packet to the State Survey Agency. The HCFA-116 information is then entered into the CLIA data system. The date of the data entry becomes the participation date (the first day that testing may begin). A laboratory can not begin patient testing until a CLIA Certificate has been obtained. Laboratory billing for Medicare and/or Medicaid can not be any earlier than the participation date.

The HCFA Data System

The HCFA Data System maintains files on all CLIA certificate. It contains the Online Survey Certification and Reporting (OSCAR) System; the Online Data Input and Edit (ODIE) System; and the CLIA Data Base. The CLIA database maintains and stores data pertinent to the HCFA-116, CLIA certificate history, and accounting information.

The OSCAR/ODIE database maintains and stores data for surveys and proficiency testing results, plus generates reports based on data held in all three systems. All certificates and fee coupons are generated and issued through the HCFA Data System.

Fee coupons are mailed one (1) year prior to the expiration date of Certificate of Compliance renewals; fee coupons are mailed six (6) months prior to the expiration date of Certificate of Waiver and PPMP Certificate renewals. Certificates (if fees have been paid in full) are mailed one (1) month prior to the expiration date of a current certificate. Replacement certificates can be obtained from the Regional Offices.

If after two rebills a laboratory has not paid their CLIA fees, the HCFA data system automatically terminates the CLIA certificate. This information is sent to Medicare and Medicaid and a laboratory will not be paid for Medicare and Medicaid laboratory services after the certificate expiration date.

Certificate of Waiver or PPMP Certificates

Once the State Agency or Regional Office has entered the HCFA-116 into the system, a fee coupon is generated the next day and mailed. A flat fee is issued for a Certificate of Waiver ($150) and a Provider-Performed Microscopy

Procedures (PPMP) certificate ($200). Payment must be sent to a bank lock-box in Atlanta, Georgia. Upon receipt of payment, the payment is credited to the laboratory's account and authorization is sent to the HCFA contractor to issue and mail the certificates. Both certificate types are renewed every two years.

Certificate of Compliance (COC) – Certificate of Accreditation (COA)

If a laboratory requests a COC (survey by the State Survey Agency) or COA (survey by a private accrediting agency), the process is slightly different. The HCFA-116 data is entered into the data system, indicating either a COC or COA. If the application is for a COA, the laboratory will be assessed a user fee for a Registration Certificate and accreditation/validation user fee. This fee is paid by all accredited facilities whether they receive a Validation Survey or not.

Note: *The Validation Fee is 5% of the compliance (survey) fee if the State Survey Agency had conducted the survey. This fee covers the cost of Validation Surveys conducted by the State Survey Agency.] In addition, the State Survey Agency may request confirmation of accreditation status. If the application is for a COC, the laboratory will be issued a user fee for a Registration Certificate and the compliance (survey) fee.*

Payment must be sent to the bank lock-box in Atlanta, Georgia. Upon receipt of payment, the payment is credited to the laboratory's account and authorization is sent to the HCFA contractor to issue and mail the Registration Certificate. The Registration Certificate registers a laboratory and allows them to begin testing. It speaks nothing to the quality of laboratory testing. This certificate is good for two years or until a survey has been completed. This two-year time frame allows the State Survey Agency to conduct an onsite survey to assess facility compliance. It also provides HCFA the time to verify with the accreditation agency that the facility is actually accredited and a survey has been conducted.

If a laboratory applies for a COC, the State Survey Agency will contact the laboratory to set up a survey date for the initial survey. Surveys cannot be performed until the compliance fee has been paid. The survey is usually performed 3 – 6 months after the laboratory's registration certificate effective date. The initial survey date establishes the "Effective Date of Compliance" and will establish future survey dates (recertification). Upon completion of the survey, the survey information is entered into the data system and a fee coupon is generated for the issuance of the Certificate of Compliance. Upon receipt of payment, the HCFA contractors prepare and mail out the certificate.

If a laboratory applies for a COA, the survey is coordinated between the laboratory and accreditation agency. Once the survey has been completed, the accreditation agency will enter this data into the CLIA database. This verifies that the laboratory is actually accredited and also establishes the "Effective Date of Accreditation". Fees and certificates will be issued based on this date and renewed every two years. The Certificate of Accreditation is issued upon receipt of the appropriate certificate/validation fee

Validation/Complaint Investigations

Validation surveys are conducted to assess a continued deemed status of an accreditation agency under CLIA. Complaint investigations are conducted to determine the validity of the complaint and if any CLIA conditions are not met. HCFA authority to conduct validation and complaint surveys is found in 42 Code of Federal Regulations (CFR) Section 493.563. If HCFA should conduct a validation inspection, the laboratory must:

- Allow the accreditation agency to release to HCFA a copy of its most recent inspection and related correspondence;
- Allow HCFA or its agent to conduct the survey;
- Provide HCFA or its agent full access to the facility, equipment, materials, records and information and provide copies of information requested during the survey process; and
- Allow HCFA to monitor correction of any deficiencies found through the inspection process.

The basis for HCFA surveys is the outcome-oriented survey process. The survey may be either comprehensive (reviewing all CLIA Conditions) or focused (reviewing a specific condition or conditions). If HCFA or their agent substantiates a complaint allegation and finds condition-level deficiencies, then a full inspection of the laboratory is conducted.

What is the Accrediting Agencies' Relationship to HCFA?

Accrediting Agencies (AA), i.e., ASHI, are private, non-profit accreditation organizations, with requirements that are equal to or more stringent than the CLIA condition-level requirements, which have applied for deeming authority for CLIA. The AA must submit their requirements to HCFA for evaluation against the CLIA requirements. HCFA, together with CDC, evaluates the AA requirements and compares them to the CLIA condition-level requirements and administrative policies. If these federal agencies agree that the AA requirements are equal to or are more stringent than CLIA, the AA is approved as a deeming authority for a period of 2 – 6 years. This approval is published in the Federal Register, with the publication date as the effective date of the "deeming authority" or approval. Laboratories granted accreditation by an approved agency are deemed to meet the CLIA requirements, however, accredited laboratories must also meet the CLIA requirements.

At the time of the evaluation, HCFA and the AA enter into agreements as to how the administrative aspects of CLIA will be carried out between HCFA and the AA. The AA agrees to maintain the CLIA database with current information as to total volumes, specialties, subspecialties and demographic information on their laboratories. However, changes regarding laboratory ownership, name, location and director and technical supervisor (moderate and high complexity labs only) must be reported by the laboratory within 30 days directly to HCFA or the State Agency who will notify the carrier or fiscal intermediary (FI). The AA has no relationship or reporting responsibility to the carriers or FI's.

HCFA has a regulatory responsibility to monitor the AA performance and consider this performance in the reapproval process. As a part of this monitor, HCFA is authorized by statute to perform validation surveys. HCFA regulations authorize 5% of AA surveys to be validated. This means that within 90 days of the AA survey, HCFA or HCFA's Agent (SA) will perform a CLIA survey and assess the laboratory's compliance with the CLIA regulations. The results of the survey are

reported to HCFA Central Office who conducts a comparison of the validation and AA surveys for agreement, and determines a disparity rate. By regulation, the disparity rate cannot exceed 20%, or a full deeming authority review is initiated. Based on the validation comparison evaluation, HCFA provides Congress with an annual report of the validation survey results for all AAs.

AAs have no authority for enforcement of CLIA sanctions. They have their own enforcement and/or sanction protocols. Although HCFA maintains a Proficiency Testing (PT) database, AAs are required to monitor PT performance and take appropriate action as agreed during the AA's review and approval process.

What are the Accredited Laboratories Responsibilities?

Under 42 CFR 493, Subpart E, sections 493.551 to 493.575, HCFA outlines the requirements and responsibilities of Accreditation [and Exemption (under an approved State Laboratory Program)] under the CLIA program. Section 493.551 states that HCFA may deem (grant deemed status) a laboratory to meet all applicable CLIA program requirements through accreditation by a private nonprofit accreditation program if certain conditions are met:

- The regulations of the accreditation agency are equal to or more stringent than CLIA condition-level requirements and that the accredited laboratory must be in compliance with the CLIA condition-level requirements if inspected by HCFA and/or its agents (State Survey Agency).
- The accreditation program is approved under HCFA. ASHI received their HCFA approval on November 3, 1994.
- The laboratory must allow the release of information to HCFA by the accreditation program and permit HCFA inspections.

In addition to meeting accreditation standards, accredited laboratories must comply with all the CLIA regulations. Accreditation does not exclude a laboratory from meeting the CLIA regulations. To meet CLIA requirements through accreditation, a laboratory must:

- Treat proficiency testing samples in the same manner as patient sample;
- Meet notification requirements under section 493.63:
  - Notify HCFA and the approved accreditation program within 30 days of any changes in ownership, name, location, and/or director;
  - Notify the accreditation program no later than 6 months of performing any addition of a test or examination within a specialty or subspecialty that is not included in the laboratory's accreditation, so that the accreditation organization can determine compliance;
  - HCFA also requires notification of any additions and deletions of tests so that a laboratory's fees can be reassessed to reflect current status and a new certificate of accreditation can be issued.
- Comply with the requirements of the approved accreditation program;
- Permit random sample validation and complaint inspections;
- Permit HCFA to monitor the correction of any deficiencies found through HCFA inspections;
- Authorize the accreditation program to release to HCFA the laboratory's inspection findings for validation or complaint investigations;
- Authorize the Proficiency Testing program to release the laboratory's proficiency testing results to HCFA;
- Obtain a Certificate of Accreditation as required in subpart D and pay the applicable fees as required in subpart F of the CLIA requirements.
- Accept a full HCFA review to determine compliance (42 CFR Section 488.11), if a laboratory fails to meet the requirements listed above (failure to meet accreditation requirements) or in the event of a non-compliance determination resulting from HCFA validation or complaint inspection. The laboratory may be subject to suspension, revocation, limitation of the laboratory's certificate of accreditation or certain alternative sanctions and suspension of Medicare and/or Medicaid payments.
  NOTE: *More specific information can be found in 42 CFR 493.61 of the CLIA regulations.*

What is HCFA's perspective on evaluation of Proficiency Testing, Quality Control and Quality Assurance?

Proficiency Testing (PT)

Accredited laboratories must permit the PT agency to release their results and interpretations to the AA. Any laboratory that refuses to allow this release of results is no longer deemed to meet the CLIA conditions and will be subject to full review by HCFA. Likewise, if an accredited laboratory has demonstrated unsuccessful PT, the AA must notify HCFA of the PT results and the actions taken by the AA within 30 days of the initiation of such action(s). HCFA may, on the basis of the notification take an adverse action against the laboratory.

Any laboratory, which demonstrates unsuccessful performance (2 consecutive or 2 out of 3 unsatisfactory testing events), is subject to sanction action by the accreditation organization. Each deemed AA must specify the actions it takes to ensure the laboratory corrects the cause(s) of the PT failures. The AA action must be equivalent to those of HCFA. Current HCFA policy requires the laboratory to undertake corrective action of technical assistance and training for its first unsuccessful performance. The laboratory must demonstrate satisfactory (80% or better) performance on the next PT event.

If the laboratory performs unsatisfactorily in the next event(s), (the second unsuccessful), the laboratory is required to stop testing in the analyte(s), specialty, or subspecialty of failure and demonstrate two consecutive satisfactory events before it can resume testing.

During a CLIA survey, part of evaluating a laboratory's PT performance includes an evaluation of any unacceptable results(s) and the laboratory's corrective action. The surveyor looks for documentation to assure the laboratory has reviewed quality control, calibration, instrument maintenance, corrective action for out-of-control results, test performance, and adherence to the laboratory's policies and procedures in determining the corrective action needed. The laboratory is also required to monitor the corrective action for effectiveness through Quality Assurance.

For ungraded results, the laboratory should evaluate their results against the expected results and determine if they would have performed satisfactorily. Documentation of this evaluation must be maintained for two years. If a laboratory is enrolled in PT for unregulated analytes, this will meet the Quality Assurance requirements to assure accuracy twice a year. During a survey, the surveyor will assure there has not been two consecutive ungraded events, and if there has been, the surveyor reviews the laboratory's performance.

The AA [see section 493.557(a)(12)] must report accredited laboratories that demonstrate unsuccessful performance, for regulated analytes listed in subpart I, to HCFA. Any laboratory found to have referred PT samples to another laboratory for testing must have its accreditation denied and HCFA must be notified of the denial. Referral of PT samples requires HCFA, by statue, to revoke the laboratory's CLIA certificate for a minimum of one year. HCFA has no discretion regarding PT referral.

The purpose of PT is to provide a snapshot in time of the laboratory's quality. PT samples should be handled in the same manner as patient samples. The laboratory should perform no special instrument maintenance nor utilize special personnel when testing PT samples. PT provides an indication of the quality of patient testing and offers the laboratory an opportunity to assess its Quality Control (QC) and Quality Assurance (QA) activity. Unsuccessful PT results may be indicators that QC or QA activity needs revision, which can be the case as instruments age, new instruments are placed into service, new employees hired or other changes occur which may affect quality.

PT participation and performance is intended to be educational and not punitive. However, if a laboratory demonstrates unsuccessful; performance in 2 consecutive or 2 out of 3 testing events, the causative problems have existed for 8-12 months without identification and correction through a laboratory's QA process. This indicates a potential for jeopardizing patient testing quality and reliability.

Quality Control

Quality Control (QC) is the means by which a laboratory validates and monitors the accuracy of its patient test results on a day to day basis, and is a means, which allows the laboratory to detect error or potential sources of analytical error. However, HCFA realizes that to accomplish the outcome goal of accurate results, the QC program must be developed with all the unique laboratory factors in mind such as equipment, volume, methods, personnel, patient distribution, urgency of results, etc.. Therefore, surveyors review the laboratory's policies and procedures and QC records to assure the laboratory's stated QC goals can be realized by the established policies and procedures the laboratory has developed. Surveyors also evaluate QC results as they relate to PT results and events.

Method validation or verification is also part of QC. This does not only include in-house developed methods, but also newly implemented high complexity FDA approved methods as well as modified, moderate complexity FDA approved methods. Documentation of validation or verification needs to be maintained as long as the method is in use or two years after it is discontinued.

Quality Assurance (QA)

Quality Assurance is the system the laboratory has developed and put into place, which assures analytical accuracy and compliance with the laboratory, established policies and procedures and the CLIA regulations. The QA program should assure and document that the laboratory's stated goals for all the conditions of CLIA are met, and that when problems or outcomes (possibly adverse) are identified, they are investigated, resolved and monitored for successful resolution. The QA system ensures that the policies and procedures are appropriate for adequate monitoring and correction of problems and are effective in preventing recurrences of any identified problems.

In CLIA, the ten QA standards encompass the entire CLIA regulation. The ten QA standards are monitors of the following CLIA conditions: Patient Test Management (Subpart J), Quality Control (Subpart K), Proficiency Testing (Subpart I), Personnel (Subpart M), General Provisions (Subpart A), and Quality Assurance (Subpart P). If the laboratory has defined an effective QA system, which evaluates and monitors the ten QA standards, then all conditions of CLIA should be met.

What is HCFA's perspective on evaluation of Conditions, Immediate Jeopardy and Standard level deficiencies?

Conditions

HCFA's authority to impose sanctions on all laboratories is found in Subpart R, 42 CFR 493. The Conditions of CLIA must be met for a laboratory to be in compliance with CLIA. A laboratory that has systemic or pervasive quality problems is determined to be out of compliance with CLIA. Sentinel events can also cause a laboratory to be out of compliance with CLIA if they are of a serious nature and have far-reaching or permanent negative effects.

A laboratory can be found out of compliance under two situations: condition level deficiencies or condition level deficiencies with immediate jeopardy (IJ). When HCFA or its agent determines a laboratory to be out of compliance with the CLIA conditions, the laboratory has demonstrated failure to meet the requirements for certification. A laboratory must correct condition level non-compliance within 90 days or sanction actions will be proposed by HCFA. (42 CFR 493.1814).

Immediate Jeopardy

The same definition applies for Immediate Jeopardy (IJ) except that in this case, HCFA or its agent has determined that the laboratory's noncompliance with condition level deficiencies demonstrates a high probability that serious harm or injury to patients could occur at any time, or already has occurred and my well occur again if patients are not protected effectively from the harm, or the threat is not removed. Under 42 CFR 493.1812(a), HCFA requires the laboratory to take immediate action to remove the jeopardy. In this case, HCFA usually directs the laboratory to suspend the service until the jeopardy has been removed. A laboratory must correct IJ within 23 days or sanction action will be proposed by HCFA. (42 CFR 493.1812)

When either condition level deficiencies or condition level deficiencies with IJ are found to exist on a validation survey, the laboratory reverts to HCFA oversight until the IJ is removed and/or the conditions are met. HCFA notifies the laboratory and the AA of this situation. Once the laboratory achieves CLIA compliance, it is returned to the AA for oversight if the AA has not withdrawn or denied the laboratory's accreditation.

NOTE: *If during an accreditation survey, the AA identified IJ, the AA must notify HCFA within 10 days of a deficiency identified.*

Standards

When a laboratory has been determined to have standard-level deficient practices, this means that a requirement of CLIA has not been met, but it is not of a serious nature. A laboratory can have standard-level deficiencies yet found to be in compliance with the CLIA conditions. However, all laboratories are required to correct standard level deficiencies within 12 months or HCFA will take steps to revoke the laboratory's certificate; HCFA has no discretion on the 12-month rule.

What Does Non-compliance Signify for Accredited Laboratories

What are the consequences of denial or revocation of Accreditation?

An accredited laboratory meets CLIA requirements through the accreditation program. As noted previously, there are certain requirements a laboratory must adhere to. If for some reason, a laboratory's accreditation has been withdrawn or revoked, the laboratory retains its Certificate of Accreditation for 45 days after the laboratory receives notice from the accreditation agency or the effective date of any action taken by HCFA, whichever is the earlier date. It is the responsibility of the accrediting agency and laboratory to inform HCFA of this. The laboratory has several options which includes: change to a lower certificate type; stop laboratory testing; apply for and obtain a COC, or obtain a Registration Certificate and apply for accreditation through another accreditation organization. Once this has been determined, the certificate/billing process starts again, based on the changes.

HCFA's authority to conduct validation and complaint surveys is found in 42 CFR 493.563. Under 42 CFR 493.567, it defines the action HCFA will initiate if a laboratory refuses to cooperate and allow the survey. The laboratory may be subject to:

- full review by HCFA or its agent; and/or
- suspension, revocation, or limitation of its certificate of accreditation;

However, if a facility withdraws its prior refusal and complies with the requirements under 42 CFR section 493.563, the validation/complaint survey will resume. In additions, actions to delay or hamper the HCFA survey process result in the same outcome, as refusal of the survey. If a laboratory is found out of compliance with CLIA conditions, it is subject to the same survey and enforcement processes (principal and alternative sanctions found in 42 CFR 493.1806) applied to laboratories that are not accredited.

Revocation

HCFA may propose revocation of a CLIA certificate based on continued condition-level non-compliance which exceeds 90 days, Immediate Jeopardy which is not removed within 23 days, referral of Proficiency Testing specimens or deficiencies which are not corrected within 12 months.

There is due process prior to the revocation taking effect, and that due process differs based on the reason for revocation. The laboratory is always aware of the proposed HCFA actions and has an opportunity to respond with evidence as to why action should not be taken. The State Operations Manual sets forth HCFA's policies and procedures for due process and gives guidelines for HCFA decisions.

Once a CLIA certificate has been revoked, the owner and/or director cannot own or direct a laboratory for two years. The name of the laboratory goes on the annual CLIA Registry, which is available on the Internet at www.hcfa.gov/medicaid/clia/cliahome.htm.

Denial

HCFA may deny a CLIA Certificate of Registration, Waiver or PPM based on the information supplied in the application (HCFA-116), additional information requested, or refusal on behalf of the laboratory to submit to HCFA the requested information needed to determine compliance with CLIA requirements for a Certificate of Registration, Waiver or PPM. If, based on the application, HCFA has substantial reason to believe a laboratory could not meet the conditions of CLIA, a Certificate of Registration, Waiver or PPM can be denied, and the laboratory is notified in writing of the denial with reason for the denial. Once denied, a laboratory may resubmit an application when the reason for the denial has been remedied.

Author Index

Patrick W. Adams, MS, CHS
Ohio State University Hospital
Department of Surgery
410 W 10th Ave
N 919 Doan Hall
Columbus, OH 43210
(614) 293-8554
FAX: (614) 293-8287
E-Mail: adams-5@medctr.ohio-state.edu

Sue Bassinger
University Hospital
2211 Lomas, NE
Albuquerque, NM 87106
(505) 277-4784
FAX: (505) 277-7224

Lee Ann Baxter-Lowe, PhD, dip.ABHI
UCSF/Immunogenetics & Transplantation Laboratory
Box 0508
San Francisco, CA 94143-0508
(415) 476-6058
FAX: (415) 476-0379
E-Mail: leeannb@itsa.ucsf.edu

Ann B. Begovich, PhD
Roche Molecular Systems
1145 Atlantic Ave
Alameda, CA 94501
(510) 814-2916
FAX: (510) 522-1285
E-Mail: Ann.Begovich@Roche.com

Anne C. Belanger, MA, MT(ASCP)
Healthcare Standards Consultants
2South723 Route 59, Ste 86
Warrenville, IL 60555-1442
(630) 876-6084
FAX: (630) 876-6084
E-Mail: abelanger@msn.com

Paula Howell Blackwell, BS, CHS, MBA
10506 Bar D Trail
Helotes, TX 78023-4057
(210) 567-5697
FAX: (210) 567-4549
E-Mail: blackwell@uthscsa.edu

Cynthia E. Blanck, PhD
3714 Huntington Drive
Amarillo, TX 79109
(806) 358-1252
FAX: (806) 354-5887
E-Mail: CEBlanck@aol.com

Robert A. Bray, PhD, dip.ABHI
Emory University Hospital
Dept of Pathology, Rm F-149
1364 Clifton NE
Atlanta, GA 30322
(404) 712-7317
FAX: (404) 727-1579
E-Mail: rbray@emory.edu

Teodorica Bugawan, BS
Roche Molecular Systems
1145 Atlantic Ave
Alameda, CA 94501
(510) 814-2909
FAX: (510) 814-2910
E-Mail: teodorica.bugawan@roche.com

Mike Bunce
Oxford Transplant Center
Tissue Typing Lab
Churchill Hospital
Oxford, OX3 7LJ
United Kingdom
01865226102
FAX: 01865226162
E-Mail: mbunce@hgmp.mrc.ac.uk

Esther-Marie Carmichael, MT(ASCP), CLS, PHM
Health Care Financing Administration
Division of State Operations
75 Hawthorne Street, 4th Floor
San Francisco, CA 94105
(415) 744-3729
E-mail: ecarmichael@hcfa.gov

Mary N. Carrington, PhD, MS
NCI-FCRDC
PO Box B
Bldg 560
Frederick, MD 21702
(301) 846-1390
FAX: (301) 846-1909
E-Mail: carringt@fcrfv2.ncifcrf.gov

Pam Chapman
Emory University Hospital
HLA Lab
1364 Clifton Rd NE
Atlanta, GA 30322
(404) 712-7365

Mary Ethel Clay, MS, MT(ASCP)
University of Minnesota Medical School
420 Delaware St SE
Box 198 UMHC Mayo
Minneapolis, MN 55455
(612) 626-1905
FAX: (612) 624-5411

Myra Coppage, MS, CHS
University of Rochester Medical Center
601 Elmwood Ave
Box 8410-Surg Rm 2-8115
Rochester, NY 14642
(716) 275-0985
FAX: (716) 271-7929
E-Mail: MyraCoppage@urmc.rochester.eduer.edu

Todd Young Cooper, MT(ASCP), CHS
University of Texas Medical Branch
301 University Blvd
RSH B804B
Galveston, TX 77550-0178
(409) 747-9550
FAX: (409) 747-9555
E-Mail: tcooper@utmb.edu

Deborah O. Crowe, PhD, dip.ABHI
DCI Lab
Trans Immuno, Ste 322
1601 23rd Ave S
Nashville, TN 37212
(615) 321-0212
FAX: (615) 321-4880
E-Mail: deborah.crowe@nashlab.dciinc.org

Agustin P. Dalmasso
University of Minnesota
Laboratory Medicine and Pathology
Box 198 Mayo
420 Delaware St SE
Minneapolis, MN 55455
(612) 625-9171

Julio C. Delgado
Brigham & Women's Hospital
75 Francis St
Boston, MA 02115
(617) 632-3346
FAX: (617) 632-4466

Mary L. Duenzl
Emory University Hospital
HLA Lab
1364 Clifton Rd NE
Atlanta, GA 30322
(404) 712-7365

Brian Duffy, MA, CHS
Barnes-Jewish Hospital
HLA Lab, One Barnes Plaza
St Louis, MO 63110
(314) 747-0435
FAX: (314) 362-4647
E-Mail: bdduff@aol.com

David D. Eckels, PhD, dip.ABHI
Blood Research Inst
PO Box 2178
Milwaukee, WI 53201-2178
(414) 937-6310
FAX: (414) 937-6284
E-Mail: ddeckels@bcsew.edu

Gail Eiber, MT
North Central Blood Services
Neutrophil/Platelet Serology Lab
American Red Cross
100 South Robert St
St Paul, MN 55107
(651) 291-6797
E-Mail: Eiber@usa.redcross.org

Marcelo Fernandez-Vina, PhD, dip.ABHI
American Red Cross
National Histo Lab
22 S Green Street, Box 173
Baltimore, MD 21201
(410) 328-2522
FAX: (410) 328-2967
E-Mail: vinam@usa.redcross.org

Soldano Ferrone
Dept Mico/Immunology
New York Medical College
Basic Science Bldg Rm 308
Valhalla, NY 10595
(914) 493-8481 and (914) 594-4175

Donna M. Fitzpatrick, CHS
Massachusetts General Hospital
32 Fruit St, WHT 544
Boston, MA 02114
(617) 726-3722
FAX: (617) 724-3331
E-Mail: dfitzpatrick@partners.org

Marilena Fotino, MD, dip.ABHI
Rogosin Inst
430 E 71st St
New York, NY 10021
(212) 772-6700
FAX: (212) 861-9473

Anne Fuller
University of Utah Hospital
50 North Medical Dr, AR SOM 121
Salt Lake City, UT 84132
(801) 581-3116

Zenaida P. Gantan, MD
Irwin Memorial Blood Centers
270 Masonic Ave
San Francisco, CA 94118
(415) 567-6400
FAX: (415) 775-3859

Howard M. Gebel, PhD, dip.ABHI
LSU Medical Center
1501 Kings Highway
Shreveport, LA 71130-3932
(318) 675-6112
FAX: (318) 675-6358
E-Mail: hgebel@lsuhsc.edu

Barbara Braun Griffith
Molecular Diagnostics Cor UNM Health Science Center
915 Camino de Salud NE, BMSB Rm 332
Albuquerque, NM 87131-5301
(505) 272-4783
FAX: (505) 272-9038
E-Mail: bgriffit@salud.unm.edu

F. Carl Grumet, MD
Stanford University
800 Welch Rd
Palo Alto, CA 94304
(650) 723-7976
FAX: (650) 725-4470
E-Mail: grumet@stanford.edu

Leigh Ann Guthrie
Fred Hutchinson Cancer Research Center
428 W 21st Ave
Spokane, WA 99203
(509) 624-7728
FAX: (509) 624-7728
E-Mail: lguthrie@fhcrc.org

Martin Gutierrez
6166 Montgomery Rd.
Elkridge, MD 21075
(410) 328-2974
FAX: (410) 328-9156
E-Mail: gutierrezm@usa.redcross.org

Julia A. Hackett, BS, HS(ABHI)
National Inst of Health
13276 Musicmaster Dr
Silver Spring, MD 20904
(301) 496-8852
FAX: (301) 480-0526
E-Mail: jhackett@dtm.cc.nih.gov

Amy B. Hahn, PhD, dip.ABHI
Albany Medical College Trans Immunology
47 New Scotland Ave
Rm ME524 Mail Code 62
Albany, NY 12208
(518) 262-5574
FAX: (518) 262-6274
E-Mail: hahna@mail.amc.edu

Charles William Hamrick, CHS, CLS
3057 Maplewood Pl
Escondido, CA 92027
(619) 642-4774
FAX: (619) 642-0595
E-Mail: chamrick@ucsd.edu

John A. Hansen, MD
Fred Hutchinson Cancer Research Center
1100 Fairview Ave N
PO Box 19024
Seattle, WA 98109-1024
(206) 667-5111
FAX: (206) 667-5255
E-Mail: jhansen@fhcrc.org

Leah N. Hartung
ARUP Institute for Clinical and Experimental Pathology, LLC
500 Chipeta Way
Salt Lake City, UT 84108-1211
(801) 584-5208
E-mail: hartunln@arup-lab.com

Sandra Helman, PhD, dip.ABHI
Medical College of GA
1120 Fifteenth St
BAS 1641
Augusta, GA 30912-4091
(706) 721-3311
FAX: (706) 721-2709
E-Mail: shelman@mail.mcg.edu

Patrice K. Hennessy, CHT
Ohio State University
410 W 10th Ave
N-935 Doan Hall
Columbus, OH 43210
(614) 293-8554
FAX: (614) 293-8287
E-Mail: hennessy-1@medctr.osu.edu

Nancy F. Hensel, CHS, MT(ASCP)
Nat'l Inst of Health Hematology Branch
9000 Rockville Pike
Bldg 10 Rm 7C103
Bethesda, MD 20892-1652
(301) 402-3296
FAX: (301) 496-8396
E-Mail: henseln@nih.gov

Susie E. Herbert
Ochsner HLA Lab
2606 Jefferson Hwy
New Orleans, LA 70121
(504) 842-3769
FAX: (504) 842-2357

Debra D. Hiraki, PhD
Stanford University Stanford Blood Center
800 Welch Rd
Palo Alto, CA 94304
(415) 725-4478
FAX: (415) 725-4470

Joan E. Holcomb, MS, CHS
Emory University Hospital
HLA Lab Room C184
1364 Clifton Rd NE
Atlanta, GA 30322
(404) 712-7365
FAX: (404) 712-4717
E-Mail: Joan_Bray_Holcomb@Emory.org

Kathy A. Hopkins, MPH
Johns Hopkins University
Immunogenetics Lab
2041 E Monument St
Baltimore, MD 21205
(410) 955-3600
FAX: (410) 955-0431
E-Mail: hccs@erols.com

Louise M. Jacobbi
Saturn Management Services
Legacy Donor Foundation
208 Glenwood Drive
Metairie, LA 70005
(504) 835-2767
FAX: (504) 835-2069
E-Mail: ljacobbi@aol.com

Fran Keller
UCSD Immunogenetics & Transplant
9894 Genesee Ave
Suite 101
La Jolla, CA 92037
(619) 642-4774
FAX: (619) 642-0595

Carol Kosman
Georgetown University
3970 Reservoir Rd NW
E404 Research bldg
Washington, DC 20007
(202) 687-2142

Malak Kotb
University of Tennessee
956 Court Ave, Ste A202
Memphis, TN 38163
(901) 448-5924
FAX: (901) 448-7306

Shalini Krishnaswamy
Stanford University
800 Welch Rd
Suite #3NE
Stanford, CA 94304
(415) 725-4478
FAX: (415) 725-4470
E-Mail: Shalini@cmgm.Stanford.edu

Geoffrey A. Land, PhD, HCLD
Methodist Med Center
1441 N Beckley Ave
Dallas, TX 75203
(214) 947-3584
FAX: (214) 947-3598
E-Mail: galand@flash.net

Lauralynn K. Lebeck, PhD, MS, dip.ABHI
University of California San Diego
9894 Genesee Ave, #101
La Jolla, CA 92037
(858) 642-4774
FAX: (858) 642-0595
E-Mail: llebeck@ucsd.edu

Jar-How Lee, PhD, dip.ABHI
One Lambda, Inc Res Dept
21001 Kittridge St
Canoga Park, CA 91303-2801
(818) 702-0042
FAX: (818) 702-6904
E-Mail: jlee@onelambda.com

M. Sue Leffell, PhD, ABMLI, dip.ABHI
Johns Hopkins University
2041 E Monument St
Baltimore, MD 21205-2222
(410) 614-8976
FAX: (410) 955-0431
E-Mail: msleffel@jhmi.edu

William M. LeFor, PhD
LifeLink Foundation, Inc.
Trans Immuno Lab
409 Bayshore Blvd
Tampa, FL 33606
(813) 253-3866
FAX: (813) 254-3367
E-Mail: lefor@lifelinkfound.org

Nufatt Leong
University of Rochester Med Center Tissue Typing Lab
601 Elmwood Ave
Box Surgery
Rochester, NY 14642
(716) 275-0985
E-Mail: nufatt_leong@urmc.rochester.edu

Jimmy Loon
One Lambda, Inc
21001 Kittridge St
Canoga Park, CA 91303
(818) 702-0042
FAX: (818) 702-6904
E-Mail: jloon@onelambda.com

David F. Lorentzen
University of Wisconsin Hospitals & Clinic
600 Highland Ave
HLA - Moleculor Diagnostics
Madison, WI 53792
(608) 263-8808
FAX: (608) 263-1568
E-Mail: df.lorentzen@hosp.wisc.edu

Patrizia Luppi
University of Pittsburgh Histocompatability Center
Rangos Research Center
3460 Fifth Avenue
Pittsburgh, PA 15213-3205
(412) 692-6570
FAX: (412) 692-5809

Prema R. Madyastha, PhD
Department of Pediatric Endocrinology
Medical University of South Carolina
171 Ashley Avenue
Charleston, SC 29401
(843) 792-6807
E-Mail: madyaspr@musc.edu

Maureen P. Martin
SAIC-Frederick, NCI-RCRDC
PO Box B
Bldg 560, Rm 21-46
Frederick, MD 21702
(301) 846-1309
FAX: (301) 846-1909
E-Mail: martinm2mail.ncifcrf.gov

Paul Joseph Martin, MD
Fred Hutchinson Cancer Center
1100 Fairview Ave. N, 02-100
Seattle, WA 98109
(206) 667-4798
FAX: (206) 667-5255

Jeffrey M. McCormack, PhD, dip.ABHI
DigiScript, Inc.
381 Mallory Station Road, Suite 210
Franklin, TN 37067
(615) 778-0780
FAX: (615) 778-0781
E-Mail: jmccormack@digiscript.com

Chris Mcfarland
Fred Hutchinson Cancer Research Center
1100 Fairview Ave N
PO Box 19024
Seattle, WA 98109-1024
(206) 667-4362
FAX: (206) 667-5892

Arvind K. Menon, MS
The Rogosin Inst Immuno & Trans Center
430 E 71st St
New York, NY 10021
(212) 772-6700
FAX: (212) 861-9473

Eric Mickelson
Fred Hutchinson Cancer Center
1100 Fairview Ave N
PO Box 19024
Seattle, WA 98109-1024
(206) 667-4922
FAX: (206) 667-5255
E-Mail: emickels@fhcrc.org

Derek Middleton
Belfast City Hospital
Belfast, BT9 7TS
Northern Ireland
44 1232 263676
FAX: 44 1232 263881
E-Mail: derek.middleton@bll.n-i.nhs.uk

E.L. Milford, MD
Brigham and Women's Hospital and New England Organ Bank
Tissue Typing Lab
75 Francis St
Boston, MA 02115

Aloke Mohinen
American Red Cross
National Histo Lab
22 S Green Street, Box 173
Baltimore, MD 21201
(410) 328-2522
FAX: (410) 328-2967

Priscilla V. Moonsamy
Roche Molecular Systems
1145 Atlantic Ave
Alameda, CA 94501
(510) 814-2953
FAX: (510) 522-1285
E-Mail: Priscilla.Moonsamy@Roche.com

Beverly Muth
American Red Cross
22 S Greene St
Box 173
Baltimore, MD 21201
(410) 328-2968
FAX: (410) 328-9156

Debra K. Newton-Nash, PhD
Blood Center of Southeastern Wisconsin
PO Box 2178
Milwaukee, WI 53201-2178
(414) 937-6222
E-Mail: debra@smtpgate.bcsew.edu

Afzal Nikaein, PhD
TX Medical Specialty, Inc
7777 Forest Lane
12A South
Dallas, TX 75230
(972) 566-5794
FAX: (972) 566-3897
E-Mail: nikaein@cs.com

Brenda Nisperos
Fred Hutchinson Cancer Center
1124 Columbia St
Seattle, WA 98104
(206) 292-5768
FAX: (206) 667-5285

Charles G. Orosz, PhD
Ohio State University
1654 Upham Dr
357 Means Hall
Columbus, OH 43210
(614) 293-3212
FAX: (614) 293-4541
E-Mail: orosz-1@medctr.osu.edu

John W. Ortegel
Dept of Internal Medicine
Section of Pulmonary & Critical Care Medicine
Rush Presbyterian/St. Luke's Med Center
Chicago, IL 60612
(312) 942-2745
FAX: (312) 563-2157
E-Mail: jortegl@rush.edu

Lori Dombrausky Osowski, MS, CHS
American Red Cross
National Histocompatability Lab
22 S Greene St Box 173
Baltimore, MD 21201-1595
(410) 328-2973
FAX: (410) 328-2967
E-Mail: osowskil@usa.redcross.org

Sandra Pearson, MT(ASCP)
Health Care Financing Administration
CLIA Program
1301 Young Street, Rm 833
Dallas, TX 75202
(214) 767-4414
E-mail: spearson@hcfa.gov

Herbert A. Perkins, MD
Blood Centers of the Pacific
270 Masonic Ave
PO Box 18718
San Francisco, CA 94118-4496
(415) 749-6652
FAX: (415) 921-6184
E-Mail: hperkins@pacbell.net

Donna L. Phelan, BA, CHS, MT(HEW)
Barnes-Jewish Hosp Labs
One Barnes Plaza
St Louis, MO 63110
(314) 362-6527
FAX: (314) 362-4647
E-Mail: dlphelan@aol.com

Diane J. Pidwell, PhD MT(ASCP) dipABHI
12402 Old Harmony Landing
Goshen, KY 40026
(502) 587-4373
FAX: (502) 587-4504
E-Mail: diane.pidwell@jhhs.org

Marilyn S. Pollack, PhD, dip.ABHI
University of Texas Health Science Center
7703 Floyd Curl Dr
Dept. of Surgery
San Antonio, TX 78229-3900
(210) 567-5697
FAX: (210) 567-4549
E-Mail: pollack@uthscsa.edu

Lisa Ratner-Rothstein
Brigham & Women's Hospital Tissue Typing Lab
75 Francis St
Boston, MA 02115
(617) 732-5872

Elaine F. Reed, PhD, dip.ABHI
UCLA Immunogenetics Center
Dept. of Pathology
950 Veteran Ave
Los Angeles, CA 90095
(310) 825-7651
FAX: (310) 206-3216
E-Mail: ereed@mednet.ucla.edu

Nancy Reinsmoen, PhD, dip.ABHI
Duke University Medical Center
Box 3712
Research Park III
Durham, NC 27710
(919) 684-3089
FAX: (919) 684-9089
E-Mail: reins001@mc.duke.edu

Laura Roberts
St Francis Hospital
Histocompatibility Lab
6161 South Yale Avenue
Tulsa, OK 74136
(918) 494-6569
FAX: (918) 494-1603
E-Mail: ldroberts@saintfrancis.com

Anthony L. Roggero, CHS, CHT, MT(ASCP)
Louisianna State Universityersity Medical Center
1501 Kings Hwy
Rm 3-204
Shreveport, LA 71130
(318) 675-6115
FAX: (318) 675-4243
E-Mail: arogge@lsumc.edu

William A. Rudert, MD, PhD
University of Pittsburgh
3705 Fifth Ave
Pittsburgh, PA 15213
(412) 692-6572
FAX: (412) 692-5809

Nancy Setsuko Sakahara, BS, MT(ASCP)
Irwin Memorial Blood Centers Scientific Services
270 Masonic Ave
San Francisco, CA 94118
(415) 567-6400 x446
FAX: (415) 775-3859

Patti Samuels Saiz, CHS, CHT
Pinehurst Apartments
12301 N. McArthur # 407
Oklahoma City, OK 73142
(405) 271-7647
FAX: (405) 271-7332
E-Mail: PLSaiz@aol.com

Doreen Sese
Brigham & Women's Hospita
75 Francis St
Boston, MA 02115
(617) 738-4650
FAX: (617) 566-6176

Alan R. Smerglia
Cleveland Clinic Allogen Labs
9500 Euclid Ave
C100
Cleveland, OH 44195-5131
(216) 444-6583
FAX: (216) 444-8261
E-Mail: ars@tt.ccf.org

Anajane G. Smith, MA
Fred Hutchinson Cancer Center
1100 Fairview Ave N
PO Box 19024
Seattle, WA 98109-1024
(206) 667-5743
FAX: (206) 667-5285
E-Mail: asmith@fhcrc.org

Peter Stastny, MD
UT Southwestern Medical Center
5323 Harry Hines Blvd
MC 8886
Dallas, TX 75235
(214) 648-3556
FAX: (214) 648-2949
E-Mail: ps@sta08.swmed.edu

Lori Steiner
Roche Molecular Systems
1020 Atlantic Ave
Alameda, CA 94501
(510) 814-2924
FAX: (510) 814-2910
E-Mail: Lori.Steiner@roche.com

Linda Stempora
5735 S. Meade
Chicago, IL 60638
011-41-794757787
E-Mail: lstempora@yahoo.com

Dod Stewart, BS, CHS
Ochsner HLA Lab
2606 Jefferson Hwy
New Orleans, LA 70121
(504) 842-3027
FAX: (504) 842-2357
E-Mail: dstewart@ochsner.org

Douglas Michael Strong, PhD, MT(ASCP), BCLD
Puget Sound Blood Center
921 Terry Ave
Seattle, WA 98104
(206) 292-1889
FAX: (206) 292-8030
E-Mail: dmstrong@psbc.org

Nicole Suciu-Foca, PhD, MS, BS
Columbia University
630 W 168th St
Dept of Pathology PS 14-401
New York, NY 10032
(212) 305-6941
FAX: (212) 305-3429
E-Mail: ns20@columbia.edu

Anat R. Tambur, DMD, PhD, dip.ABHI
6720 Karlov Ave.
Lincolnwood, IL 60646
(312) 942-2054
FAX: (312) 942-6965
E-Mail: atambur@rush.edu

Gary A. Teresi, MT, CHS
Allogen Laboratories
Cleveland Clinic Found-C100
9500 Euclid Ave
Cleveland, OH 44195
(216) 444-0384
FAX: (216) 444-8261
E-Mail: gat@tt.ccf.org

Massimo M. Trucco, MD
University of Pittsburgh Histocompatability Center
Rangos Research Center
3460 Fifth Avenue
Pittsburgh, PA 15213-3205
(412) 692-6570
FAX: (412) 692-5809

Smita Vaidya, PhD
University of Texas Medical Branch
301 University Blvd
404 8th St Rm B804F RSH
Galveston, TX 77555-0178
(409) 747-9550
FAX: (409) 747-9555
E-Mail: smita.vaidya@utmb.edu

Anne M. Ward
9000 Harry Hines Blvd
Tissue Antigen Lab
Dallas, TX 75235
(214) 358-5022

Ken Welsh
Oxford Transplant Center Transplant Immunology
Churchill Hospital
Oxford, OX3 7LJ
United Kingdom
0111865226122
FAX: 0111865225616

Thomas M. Williams, MD, dip.ABHI
University of New Mexico Health Sciences Center
915 Camino de Salud, NE
Rm 337-BRF
Albuquerque, NM 87131-5301
(505) 272- 8059x5872
FAX: (505) 272-5186
E-Mail: twilliams@salud.unm.edu

Lisa Wilmoth-Hosey
Emory University Hospital
1364 Clifton Rd, NE
Atlanta, GA 30322
(404) 712-7365
FAX: (404) 712-4717
E-Mail: lisa_wilmoth.hosey@emory.org

Carl T. Wittwer
Flow Cytometry
Department of Pathology
University of Utah Medical Center
Salt Lake City, UT 84143
(801) 581-4737

Jin Wu
University of New Mexico
Health Sci Center-BMSB Room 308
915 Camino De Salud, NE
Albuquerque, NM 87131
(505) 272-4784
FAX: (505) 272-1950
E-Mail: JWU@medusa.unm.edu

Edmond Yunis, MD
American Red Cross
180 Rustcraft Rd
NE Region, Ste 115
Dedham, MA 02026
(781) 461-2146
FAX: (781) 461-2269

Adriana Zeevi, PhD, dip.ABHI
University of Pittsburgh Medical Center
Biomedical Sci Tower Rm W1552
Lothrop & Terr Sts
Pittsburgh, PA 15261
(412) 624-1073
FAX: (412) 624-6666
E-Mail: zeevi+@pitt.edu

Standards for Histocompatibility Testing

Adopted 4/98

A – GENERAL POLICIES

A1.000 These Standards have been prepared by the Committee on Quality Assurance and Standards of the American Society for Histocompatibility and Immunogenetics (ASHI), and have been approved by the ASHI Council and CLIA.

A2.000 These Standards have been established for the purpose of ensuring accurate and dependable histocompatibility testing consistent with the current state of technological procedures and the availability of reagents.

A3.000 These Standards establish minimal criteria which all histocompatibility laboratories must meet if their services are to be considered acceptable. Many laboratories, because of extensive experience and long-established programs of reagent procurement and preparation, will exceed the minimal requirements of these Standards.

A4.000 Certain Standards are obligatory. In these instances, the Standards use the word "must." Some Standards are highly recommended but not absolutely mandatory. In these instances the Standards use words like "should" or "recommended."

A5.000 Procedures to be used in histocompatibility testing often have multiple acceptable variations. The accuracy and dependability of each procedure must be documented in each laboratory or by published data from other laboratories. Use of the ASHI Technical Manual is highly recommended as a reference procedure manual for all laboratories.

A6.000 Some procedures have sufficient documentation of effectiveness to warrant their use in clinical service even though they are not available in or obligatory for all laboratories.

A7.000 The use of the name of the American Society for Histocompatibility and Immunogenetics as certification of compliance to these Standards may only be made by laboratories which have been accredited through the ASHI accreditation process.

B – PERSONNEL QUALIFICATIONS

B1.000 A **Director/Technical Supervisor** must hold an earned doctoral degree in a biologic science, or be a physician, and subsequent to graduation must have had four years experience in immunology or cell biology, two of which were devoted to formal training in human histocompatibility testing. Credit toward this 96 weeks can be applied at the rate of 19 weeks for each year of appropriate working experience in human histocompatibility testing. The Director must have documentation of professional competence in the appropriate activities in which the laboratory is engaged. This should be based on a sound knowledge of the fundamentals of immunology, genetics and histocompatibility testing and reflected by external measures such as participation in national or international workshops and publications in peer-reviewed journals. He/she is available on site commensurate with workload at the laboratory, provides adequate supervision of technical personnel, utilizes his/her special scientific skills in developing new procedures and is held responsible for the proper performance, interpretation and reporting of all laboratory procedures and the laboratory's successful participation in proficiency testing.

B2.000 A **General Supervisor** must hold a bachelor's degree and have had three years' experience in human histocompatibility testing under the supervision of a qualified Director/ Technical Supervisor or five years of supervised experience if a bachelor's degree has not been earned. CHS (ABHI) certification is highly recommended.

B3.000 A **Histocompatibility Technologist** must have had one year of supervised experience in human histocompatibility testing, regardless of academic degree or other training and experience. It is highly recommended that they be either CHS or CHT (ABHI) certified. The term Technician is applied to trainees and other laboratory personnel with less than one year's supervised experience in human histocompatibility testing, regardless of academic degree or other training and experience.

B4.000 The size of the staff must be large enough to carry out the volume and variety of tests required without a degree of pressure which will result in errors.

B5.000 All personnel must meet the standards which are required by Federal, State and local laws.

C – GENERAL COMMENTS AND QUALITY ASSURANCE

C1.000 Facilities

C1.100 Laboratory space must be sufficient so that all procedures can be carried out without crowding to the extent that errors may result.

C1.200 Lighting and ventilation must be adequate.

C1.300 Refrigerators and freezers must be maintained at temperatures optimal for storage of each type of sample or reagent. They must be monitored daily. Recording thermometers are recommended for mechanical refrigerators or freezers. These should be coupled to alarm systems with an audible alarm where it can be heard 24 hours a day. In laboratories where liquid nitrogen is utilized for storage of frozen cells, the level of liquid nitrogen in the cell freezers must be monitored at intervals which will ensure an adequate supply at all times. Ambient temperature and/or the temperatures of incubators in which test procedures are carried out must be monitored daily to ensure that these procedures are carried out within temperature ranges specified in the laboratory's procedure manual.

C1.400 Laboratories performing mixed lymphocyte cultures, HLA-D, or cellular Class II typing should have a laminar flow hood or other appropriately aseptic work area. Counters should be standardized according to the manufacturer's instructions at regular intervals. The incubator should be monitored daily in relation to temperature (37°C) and CO_2 concentration (5% +/- 1%) and should be appropriately humidified.

C1.500 Laboratories using radioactive materials must store radioactive materials and conduct procedures using radioactive materials in a designated section of the laboratory. Radioactive materials must be disposed of at locations designated by local institutions.

C1.600 Equipment Maintenance and Function Checks

C1.610 The laboratory must establish and employ policies and procedures for the proper maintenance of equipment, instruments and test systems by 1) defining its preventive maintenance program for each instrument and piece of equipment, and by 2) performing and documenting function checks on equipment with at least the frequency specified by the manufacturer.

C1.700 Adequate facilities to store records must be immediately available to the laboratory.

C1.800 The laboratory must be in compliance with all applicable Federal, State and local laws which relate to laboratory employee health and safety; fire safety; and the storage, handling and disposal of chemical, biological and radioactive materials.

C1.900 Computer assisted analyses must be reviewed, verified and signed by the Supervisor and/or Laboratory Director before issue.

C1.910 The computer software program used for analyses must be documented.

C2.000 Specimen Submission and Requisition.

C2.100 The laboratory must have available and follow written policies and procedures regarding specimen collection.

C2.110 The laboratory must perform tests only at the written or electronic request of an authorized person. The laboratory must assure that the requisition includes: 1) the patient's name or other method of specimen identification to assure accurate reporting of results; 2) the name and address of the authorized person who ordered the test; 3) date of specimen collection; 4) time of specimen collection, when pertinent to testing; 5) source of specimen. Oral requests for laboratory tests are permitted only if the laboratory subsequently obtains written authorization for testing within 30 days of the request.

C2.120 Blood samples must be individually labeled as to the name, or other unique identification marker for the donor and the date of collection. When multiple blood tubes are collected, each tube must be individually labeled.

C2.130 The laboratory must maintain a system to ensure reliable specimen identification, and must document each step in the processing and testing of patient specimens to assure that accurate test results are recorded.

C2.140 The laboratory must have criteria for specimen rejection and a mechanism to assure that specimens are not tested when they do not meet the lab's criteria for acceptability.

C2.200 Blood samples must be obtained using a location which does not compromise aseptic techniques. The donor's skin must be prepared by a technique which ensures minimal possibility of infection of the donor or contamination of the sample. All needles and syringes must be disposable.

C2.210 All blood samples should be handled and transported in accordance with the understanding that they could transmit infectious agents.

C2.220 The anticoagulant/preservation medium used must be shown to preserve sample viability, antigens and distributions of markers/characteristics of cells tested for the (maximum) length of time and under all the specified storage conditions the laboratory permits, on the basis of documented or published stability tests, between sample collection and testing.

C2.300 Reagents.

C2.310 All reagents must be properly labeled and stored according to manufacturers' instructions. Each serum or monoclonal antibody or typing tray must be stored at a temperature appropriate to maintaining its reactivity and specificity.

C2.320 Reagents, solutions, culture media, controls, calibrators and other materials must be labeled to indicate 1) identity and when significant, titer, strength or concentration; 2) recommended storage requirements; 3) preparation and/or expiration date and other pertinent information.

C3.000 All procedures in use in the laboratory must be detailed in a procedure manual which is immediately available where the procedures are carried out. The procedure manual must be reviewed at least annually by the Director and written evidence of this review must be in the manual. Any changes in procedures must be initialed and dated by the Director at the time they are initiated.

C4.000 Quality Assurance

C4.100 The laboratory must participate in at least one external proficiency testing program, if available, in each category for which ASHI accreditation is sought.

C4.200 If a laboratory's performance in an external proficiency testing program is unsatisfactory in any category for which ASHI accreditation is sought, the laboratory must participate in an enhanced proficiency testing program in that category until performance is deemed satisfactory.

C4.300 Proficiency test samples must be tested in a manner comparable to that for testing patient samples.

C4.400 The laboratory must, at least once each month, give each individual performing tests a characterized specimen as an unknown to verify his or her ability to reproduce test results. The laboratory must maintain records of these results for each individual.

C4.500 The laboratory must establish and employ policies and procedures, and document actions taken when 1) test systems do not meet the laboratory's established criteria including quality control results that are outside of acceptable limits; and when 2) errors are detected in the reported patient results. In the latter instance, the laboratory must promptly a) notify the authorized person ordering or individual utilizing the test results of reporting errors; b) issue corrected reports, and c) maintain copies of the original report as well as the corrected report for two years.

C5.000 Records and Test Reports.

C5.100 The laboratory must maintain a legally reproduced record of each test result, including preliminary reports, for all subjects tested for a period of two years or longer, depending on local regulations.

C5.110 These records must include log books, and at least a summary of results obtained.

C5.120 Work sheets must clearly identify the subject whose cells were tested, the typing sera which were used, the date of the test and the person performing the test.

C5.130 For each cell-serum combination, the results must be recorded in a manner which indicates the approximate percent of cells killed. The numerical codes used in the ASHI Laboratory Manual are recommended.

C5.140 Reports or records, as appropriate, should include a brief description of the specimen (blood, lymph node, spleen, bone marrow, etc.) used for testing.

C5.150 Membranes or autoradiographs from nucleic acid analysis must be retained as a permanent record.

C5.160 Records may be saved in computer files only, provided that back-up files are maintained to ensure against loss of data. It is recommended that legal advice be sought to be certain that computer files meet requirements in case of legal actions.

C5.170 For marrow transplantation, the donor must give his informed consent before blood is taken for typing and before the donor is placed on a list of donors available to be called.

C5.180 For marrow transplantation, donor records should be maintained so that donors can be rapidly retrieved according to HLA type.

C5.190 The laboratory must have adequate systems in place to report results in a timely, accurate and reliable manner.

C5.200 The report should contain:

a. The date of collection of sample.
b. The Laboratory and/or Institution's unique identifiernumber.
c. The name of the individual tested.
d. The date the individual was tested.
e. The date of the report.
f. The test results.
g. Any appropriate control value/normal ranges, where appropriate.
h. Appropriate interpretations and the signature of the Laboratory Director, or designate in his/her absence.

C5.210 The laboratory must indicate on the test report any information regarding the condition and disposition of specimens that do not meet the laboratory's criteria for acceptability.

C5.220 The laboratory must maintain permanent files of all internal and external quality control tests.

C5.230 Laboratories should have a mechanism in place for resolving any tissue typing discrepancies that may occur between laboratories.

C6.000 The Laboratory Director and technical staff must participate in continuing education relative to each category for which ASHI accreditation is sought.

C7.000 An accredited laboratory may engage another laboratory to perform testing not done by the primary laboratory. In that event, the subcontracting laboratory must be accredited by the American Society for Histocompatibility and Immunogenetics, if the testing is covered by ASHI Standards. If genetic systems not covered by ASHI Standards (ABO, RBC enzymes, etc.) are subcontracted, the subcontracting laboratory must document expertise and/or accreditation in those systems. The identity of the subcontracting laboratory and that portion of the testing for which it bears responsibility must be noted in the reports.

D – HLA ANTIGENS

D1.000 Terminology of HLA antigens must conform to the latest report of the W.H.O. Committee on Nomenclature.

D1.100 Potential new antigens not yet approved by the W.H.O. Committee must have a local designation which cannot be confused with W.H.O. terminology.

D1.200 Phenotypes and genotypes should be expressed as recommended by the W.H.O. Committee, as in the following examples:

D1.210 Single antigens: HLA-B7 (or B7 if HLA is obvious from context).

D1.211 The locus designation must always be included.

D1.220 Phenotype: HLA-A2,30; B7(Bw6), 44(Bw4); Cw5; DR1,4; DQ5,7; Dw1,w4.

D1.221 If only a single antigen is found at a locus, the phenotype may include it twice only if homozygosity is proven by family studies. Conversely, a "blank antigen" can only be assigned if proven by family studies.

D1.230 Genotype:

HLA-A2,B44(Bw4),Cw5,DR1,DQ5,Dw1/A30,B7(Bw6), Cwx,DR4,DQ7,Dw4.

D2.000 Determination of haplotypes and genotypes can only be done by family studies.

D2.100 Family studies.

D2.110 All available members of the immediate family should be typed.

D2.111 Typing for HLA-A,-B locus antigens is mandatory. Typing for HLA DR is highly recommended.

D2.112 Typing for HLA-C, -D, -DQ and/or -DP may be helpful in some situations but is not mandatory.

D2.113 Reports of HLA family studies must include haplotype assignments and an explanation of recombination when this occurs.

D2.200 Unrelated Individuals.

D2.210 The probability of possible haplotypes, given the phenotype, may be determined from known haplotype frequencies in the relevant population.

D2.220 The haplotype frequencies used should be from the most complete and reliable studies available.

D2.230 The haplotype frequencies used should be those most appropriate for the ethnic group of the subject.

D2.240 Reports of probable haplotypes based on population frequencies should clearly indicate that they were so derived.

D3.000 The laboratory must have a written policy that it follows that establishes when antigen redefinition and retyping are required.

E – SEROLOGIC TYPING – HLA CLASS I

E1.000 HLA-A locus antigens.

E1.100 The laboratory must be able to type for all HLA-A specificities which are officially recognized by the W.H.O. and for which sera are readily available.

E2.000 HLA-B locus antigens.

E2.100 The laboratory must be able to type for all HLA-B specificities which are officially recognized by the W.H.O. and for which sera are readily available.

E3.000 HLA-C locus antigens.

E3.100 Typing for C locus antigens is not mandatory.

E3.200 If C locus typing is done, the laboratory should make continuing efforts to type for all C locus antigens for which sera can be obtained.

E4.000 Serologic typing techniques – HLA Class I

E4.100 Techniques used must be those which have been established to define HLA Class I specificities optimally.

E4.200 Techniques used should employ minimal amounts of rare reagents. In general, only 1 microliter of each typing serum should be used in each serological test. When monoclonal antibodies are used, the amount should be adequate to ensure accuracy of the assay employed.

E4.300 Control sera.

E4.310 Each typing must include at least one positive control serum, previously shown to react with all cells expressing Class I antigens.

E4.311 Typing results may be invalid if the positive control fails to react as expected.

E4.320 Each typing must include at least one negative control serum. The negative control should either be one previously shown to lack antibody or should be from a healthy male with no history of blood transfusion.

E4.321 Cell viability in the negative control well at the end of incubation must be sufficient to permit accurate interpretation of results. For most techniques, viability should exceed 80%.

E4.322 In assays in which cell viability is not required, results on positive and negative controls must be sufficiently discriminatory to permit accurate interpretation of results.

E4.400 Target Cells.

E4.410 Cells may be obtained from peripheral blood, bone marrow, lymph nodes or spleen, or cultured cells.

E4.411 If the cell donor has been transfused within the previous seven days, results are acceptable only if antigens are unequivocally defined, with no more than two antigens per locus.

E4.420 Typing for HLA Class I antigens may employ mixed mononuclear cells or T-lymphocyte-enriched preparations.

E4.500 Each HLA-A,B,C antigen should be defined by at least two sera, if both are operationally monospecific. If multispecific sera must be used, at least three partially non-overlapping sera should be used to define each HLA-A,B,C antigen.

E4.600 Each monoclonal antibody used for alloantigen assignment must be used at a dilution and with a technique in which it demonstrates: 1) specificity comparable to antigen assignment by alloantisera on a well-defined cell panel or 2) specificity officially recognized by the W.H.O.

E5.000 Internal Quality Control.

E5.100 Cell panels of known HLA Class I type must be available to prove the specificity of new antibodies. The panel cells should include at least one example of each HLA antigen the laboratory should be able to define, and be from a variety of ethnic groups. Storage of at least some panel cells at 80°C or in liquid nitrogen may be necessary to insure availability of required antigens.

E5.200 Typing Sera.

E5.210 It is recommended that the specificity of typing sera obtained locally be confirmed in at least one other HLA laboratory.

E5.220 Specificity of individual sera received from other laboratories or commercial sources must be confirmed to ensure that they reveal the same specificities in the receiving laboratory.

E5.230 Each lot of new commercial typing trays must be evaluated by testing either with at least five different cells of known phenotype representing major specificities or in parallel with previously evaluated trays.

E5.300 Complement.

E5.310 Each batch of complement must be tested to determine that it mediates cytotoxicity in the presence of specific antibody, but is not cytotoxic in the absence of specific antibody.

E5.311 The test should employ multiple dilutions of complement to ensure that it is maximally active at least one dilution beyond that intended for use.

E5.312 The test should be carried out with at least two antibodies which should react with at least two different test cells and at least one cell which should not react. A strong and a weak antibody should be selected for the test, or serial dilutions of a single serum may be used.

E5.313 Complement should be tested separately for use with each type of target cell, since a different dilution or preparation may be required for optimal performance.

E6.000 External quality control.

E6.100 At least one form of external quality control must be used to ensure that local definition of HLA antigens agrees with that in other laboratories.

E6.200 The external quality control may consist of comparison of results using typing sera tested by others or typing of cells typed by others. Preferably, both approaches should be used.

E6.300 External quality controls may be carried out through local or regional arrangements and by participation in the ASHI/CAP or another equally acceptable proficiency test.

F – SEROLOGIC TYPING – HLA CLASS II

F1.000 HLA-DR Region Antigens.

F1.100 Typing for DR locus antigens is highly recommended.

F1.200 If DR locus typing is done, the laboratory must be able to type for all HLA-DR specificities for which sera are readily available, and should make continuing efforts to type for all recognized HLA-DR antigens.

F2.000 HLA-DQ Region Antigens.

F2.100 Typing for DQ locus antigens is not mandatory.

F2.200 If DQ locus typing is done, the laboratory must be able to type for all HLA-DQ specificities for which sera are readily available and should make continuing efforts to type for all recognized HLA-DQ antigens.

F3.000 HLA-DP Region Antigens.

F3.100 Typing for DP locus antigens is not mandatory.

F3.200 If DP locus typing is done, the laboratory must be able to type for those HLA-DP specificities which do not have a "w" prefix, and should make continuing efforts to type for all recognized HLA-DP antigens.

F4.000 Serologic Typing Techniques – HLA Class II

F4.100 Techniques used must be those which have been established to define HLA Class II specificities optimally.

F4.200 Techniques used should employ minimal amounts of rare reagents. In general, only 1 microliter of each typing serum should be used in each serological test. When monoclonal antibodies are used, the amount should be adequate to ensure accuracy of the assay employed.

F4.300 Control Sera.

F4.310 Each typing must include at least one positive control serum, previously shown to react with all cells expressing Class II antigens.

F4.311 Typing results may be invalid if the positive control fails to react as expected.

F4.320 Each typing must include at least one negative control serum. The negative control should either be one previously shown to lack antibody or should be from a healthy male with no history of blood transfusion.

F4.321 Cell viability in the negative control well at the end of incubation must be sufficient to permit accurate interpretation of results. For most techniques, viability should exceed 80%.

F4.322 In assays in which cell viability is not required, results on positive and negative controls must be sufficiently discriminatory to permit accurate interpretation of results.

F4.400 Target Cells.

F4.410 Cells may be obtained from peripheral blood, bone marrow, lymph nodes or spleen, or cultured cells.

F4.411 If the cell donor has been transfused within the previous seven days, results are acceptable only if antigens are unequivocally defined, with no more than two antigens per locus.

F4.420 Typing for Class II antigens usually requires B lymphocyte-enriched preparations. The proportion of B lymphocytes in each preparation must be confirmed and should usually be at least 80%.

F4.421 Separation of B lymphocytes is not required if a technique is used which distinguishes between T and B lymphocytes or in assays in which antibodies with well-defined specificity are used which only define HLA class II molecules.

F4.500 Each HLA-Class II antigen should be defined by at least three sera, if all are operationally monospecific. If multispecific sera must be used, at least five partially non-overlapping sera should be used to define each HLA-Class II antigen.

F4.510 If monoclonal antibodies are used, each DR, DQ, DP antigen should be defined by at least two antibodies with private epitope specificity or one antibody with private epitope specificity and two with public epitope specificity or at least three partially non-overlapping antibodies with public epitope specificities.

F4.600 Each monoclonal antibody used for alloantigen assignment must be used at a dilution and with a technique in which it demonstrates: 1) specificity comparable to antigen assignment by alloantisera on a well-defined cell panel or 2) specificity officially recognized by the W.H.O.

F5.000 Internal Quality Control.

F5.100 Cell panels of known HLA Class II type must be available to prove the specificity of new antibodies. The panel cells should include at least one example of each HLA antigen the laboratory should be able to define, and be from a variety of ethnic groups. Storage of at least some panel cells at -80°C or in liquid nitrogen may be necessary to insure availability of required antigens.

F5.200 Typing Sera.

F5.210 It is recommended that the specificity of typing sera obtained locally be confirmed in at least one other HLA laboratory.

F5.220 Specificity of individual sera received from other laboratories or commercial sources must be confirmed to ensure that they reveal the same specificities in the receiving laboratory.

F5.230 Each lot of new commercial typing trays must be evaluated by testing either with at least five different cells of known phenotype representing major specificities or in parallel with previously evaluated trays.

F5.300 Complement.

F5.310 Each batch of complement must be tested to determine that it mediates cytotoxicity in the presence of specific antibody, but is not cytotoxic in the absence of specific antibody.

F5.311 The test should employ multiple dilutions of complement to ensure that it is maximally active at least one dilution beyond that intended for use.

F5.312 The test should be carried out with at least two antibodies which should react with at least two different test cells and at least one cell which should not react. A strong and a weak antibody should be selected for the test, or serial dilutions of a single serum may be used.

F5.313 Complement should be tested separately for use with each type of target cell, since a different dilution or preparation may be required for optimal performance.

F6.000 External Quality Control.

F6.100 At least one form of external quality control must be used to ensure that local definition of HLA antigens agrees with that in other laboratories.

F6.200 The external quality control may consist of comparison of results using typing sera tested by others or typing of cells typed by others. Preferably, both approaches should be used.

F6.300 External quality controls may be carried out through local or regional arrangements and by participation in the ASHI/CAP or another equally acceptable proficiency test.

G – MIXED LEUKOCYTE CULTURE TESTS

G1.000 Mixed Leucocyte Culture (MLC) Test

G1.100 At the start of culture, lymphocyte viability should exceed 80%.

G1.200 Serum used in the culture medium must be screened for support of cellular proliferation and the absence of cytotoxic antibodies and must be sterile.

G1.300 MLC cultures must be incubated for the length of time shown to give appropriate proliferation.

G1.400 The negative control for each responder cell must consist of responder cells stimulated with autologous cells.

G1.500 The positive control for each responder cell must consist of either of the following: a) responder cells stimulated with cells from three or more unrelated individuals; or, b) responder cells stimulated with cells from two unrelated individuals and a pool of cells from at least three other individuals.

G1.600 In each MLC test, stimulator cells must be shown to be capable of stimulating unrelated cells.

G1.700 An MLC evaluating the potential for rejection is invalid if the cells from the potential recipient do not respond to unrelated stimulator cells. An MLC evaluating the potential for graft versus host disease is invalid if cells from a potential recipient fail to stimulate unrelated cells.

H – ANTIBODY SCREENING

H1.000 Techniques.

H1.100 A complement-dependent cytotoxic technique must be used for the detection of antibody to HLA antigens. Other techniques may be used as an adjunct to the lymphocyte-based technique if they have been demonstrated by the laboratory, or established in publications, to identify HLA-specific antibody with a specificity equivalent or superior to that of the lymphocyte-based technique or at a level appropriate for the clinical indication.

H1.120 To detect antibodies to HLA Class II antigens, a method must be used that distinguishes them from antibodies to Class I antigens.

H1.130 Techniques that detect lymphocyte-dependent antibody or test for cellular sensitization may be used to supplement the laboratory's technique that meets the requirements of H1.100.

H1.140 Techniques to identify non-HLA alloantibodies such as those using monocytes or cells from specific tissues may be used to supplement the laboratory's that meets the requirements of H1.100.

H1.150 Reports of results of antibody screening must include identification of the technique.

H1.200 Sera.

H1.210 Sera must be tested at a concentration determined to be optimal for detection of antibody to HLA antigens. The dilution(s) must be documented.

H1.220 Negative control sera must include a serum from non-alloimmunized human donor(s). Each assay must include negative control(s).

H1.230 Positive control sera should be from highly alloimmunized individuals and documented to react with HLA antigens. The antibodies must be of the appropriate isotype for each assay. Each assay must include positive control(s).

H1.300 Panel.

H1.310 The panel of HLA antigens must include sufficient panel cell donors to ensure that they are appropriate for the population served and for the use of the data.

H1.320 For assays intended to provide information on antibody specificity, documentation of the Class I and Class II phenotypes of the donors of the panel cells must be provided.

H1.330 To identify the specificity of an antibody with certainty, the laboratory should test the serum with additional cells expressing and lacking the candidate antigen and cross-reactive antigens.

H2.000 Antibody screening by complement-dependent cytotoxicity

H2.100 Positive and negative controls for the activity of complement and the viability of the test cells must be included on each tray.

H2.200 Sera must be tested undiluted.

H.2.300 Target Cells.

H2.310 Target cells may be mononuclear cells from peripheral blood, lymph nodes, spleen or cell lines or CLL.

H2.320 To detect antibodies to HLA Class II antigens, B lymphocytes, B lymphoblastoid cell lines or CLL may be used.

H2.400 The specificity of serum to be used as a reagent must be validated in other laboratories. Specificity determinations should include supporting statistical analysis.

H3.000 Antibody screening by Flow Cytometry.

H3.100 Laboratories performing assays using flow cytometry must conform to the Standards in Section Q1.000 Instrument Standardization/Calibration and in Section Q2.000 Flow Cytometric Crossmatch Technique.

H3.200 If cells pooled from multiple individuals are used for a present/not present detection of antibody, the cells used must cover the major antigen specificities or CREG. The laboratory must cite the publication used to define major antigen specificities or CREG.

H3.300 For assignment of antibody specificity, cells from a sufficient number of individuals must be used to cover appropriate specificities. To assign specificity for major antigen specificities or CREG, sufficient pools of individual cells must be used. The laboratory must cite the publication used to define major antigen specificities or CREG.

H4.000 Antibody Screening by ELISA

H4.100 Laboratories using ELISA techniques for antibody screening must conform to Standards in Sections R. Enzyme Linked Immunosorbent Assay (ELISA).

H4.200 To control for non-specific binding of antibody, each serum must be assayed in a test system which lacks HLA antigen.

H4.300 Antigens obtained from pooled cells may be used for a present/not present detection of antibody. Cells from a sufficient number of individuals must be used to cover major antigen specificities. The number of individuals must be documented.

H4.400 Sera must be tested at a concentration determined to be optimal for detection of antibody to HLA antigens. The dilution must be documented.

H4.500 The panel for HLA antigens must include sufficient panel cell donors to ensure that they are appropriate for the population served and for the use of the data.

H4.510 Antigens obtained from pooled cells may be used for a present/not present detection of antibody.

H4.520 For assays intended to provide information on antibody specificity, the manufacturer must provide documentation of the Class I and Class II phenotypes of the donors of the panel cells.

I – RENAL TRANSPLANTATION

I1.000 If cadaver donor transplants are done, personnel for the required histocompatibility testing must be available 24 hours a day, seven days a week.

I2.100 Laboratories must have a documented policy in place to evaluate the extent of sensitization of each patient at the time of their initial evaluation. (This could include testing for autoantibody, DTT reducible antibody, etc.)

I2.110 Laboratories must have a program to periodically screen serum samples from each patient for antibody to HLA antigens. Samples must be collected monthly. The laboratory must have a documented policy establishing the frequency of screening serum samples and must have data to support this policy.

I2.120 Laboratories should maintain a record of potentially sensitizing events for each patient. Serum samples should be collected and stored after each of these events for possible subsequent screening for antibody to HLA antigens and/or use in crossmatch tests.

I2.200 Antibodies of defined HLA specificity should be identified and reported.

I2.300 Studies should be performed to distinguish antibodies to HLA antigens from antibodies with other specificities.

I3.000 Crossmatching.

I3.100 Crossmatching must be performed prospectively.

I3.200 Techniques.

I3.210 Crossmatching must use techniques documented to have increased sensitivity in comparison with the standard complement-dependent, basic microlymphocytotoxicity test.

I3.220 Lymphocytotoxic or flow cytometry crossmatches must be performed with potential donor T lymphocytes and should be performed with B lymphocytes.

I3.300 Samples.

I3.310 Sera must be tested at a dilution that is optimal for each assay. For lymphocytotoxicity crossmatches, sera must be tested undiluted and should be tested at one or more dilutions.

I3.320 Sera obtained 14 days after a potentially sensitizing event should be included in a final crossmatch.

I3.400 Serum samples used for crossmatching should be retained in the frozen state for at least 12 months following transplantation.

I4.000 HLA Typing.

I4.100 Prospective typing of donor and recipient for HLA-A, B, and DR antigens is mandatory.

I4.200 Typing donor and recipient for HLA-C, DQ, DP and D antigens is optional

I5.000 Family Donors.

I5.100 All available members of the immediate family should be typed for accurate haplotype assignment.

I5.200 An MLC test may be advisable before use of a family donor. Either a one-way or a two-way MLC can be used.

I5.300 Final crossmatches performed prior to transplantation should utilize a recipient serum sample collected within the past 48 hours before transplant if the recipient has class I lymphocytotoxic antibodies (reactivity with more than 15% panel cells) or has had a recent sensitizing event (see H3.120). Otherwise, a serum collected within seven days should be used.

I5.400 A reverse lymphocytotoxicity and granulocytotoxicity crossmatch (donor serum, patient cells) is advisable in mother to child pretransplant donor specific blood transfusions.

I6.000 Cadaver Donors.

I6.100 Donors may be typed using lymphocytes from lymph nodes, spleen or peripheral blood.

I7.000 Tests to monitor the immune responsiveness of a recipient are an appropriate function for a histocompatibility laboratory. These may include, but are not limited to, the following:

I7.100 Enumeration of T lymphocytes (and subsets), B cells, NK cells and monocytes.

I7.200 Evaluation of function of T cells (cytotoxic, helper and suppressor activity), B cells (antibody production), and NK cells (cytotoxicity).

J – NON-RENAL ORGAN TRANSPLANTATION

J1.000 In cases when patients are at high risk for allograft rejection (e.g., patients with histories of allograft rejection, patients with high levels of preformed class I HLA antibodies), donors and recipients should be typed for HLA-A, B and DR antigens whenever possible.

J2.000 Patients at high risk for allograft rejection should be screened whenever possible for the presence of anti-HLA-A or B lymphocytotoxic antibodies, and for autoreactive antibodies.

J3.000 Crossmatching. See Section I3.000.

J3.100 Sera from patients at high risk for allograft rejection should be prospectively crossmatched whenever possible. Techniques with increased sensitivity (see I3.130) must be used. Crossmatch results should be available prior to transplantation of a presensitized patient.

J3.200 Final crossmatches performed prior to transplantation should utilize a recipient serum sample collected within the past 48 hours before transplant if the recipient has Class I lymphocytotoxic antibodies (determined by the laboratory's established criteria for defining positive reactivity of recipient sera against donor's unseparated cells or enriched T cells) or has had a recent sensitizing event (see I3.300). Otherwise, a serum collected within seven days should be used.

J3.300 If the patient receives a blood transfusion, has an allograft that is rejected or removed, or experiences any other potentially sensitizing event, a serum sample obtained at least 14 days post-sensitization should be used in the final crossmatch.

J3.400 Whenever possible, tissues for recipients at high risk for allograft rejection should come from crossmatch-negative donors (i.e., crossmatch with unseparated lymphocytes or enriched T-cells is less than 20% above background).

K – MARROW TRANSPLANTATION

K1.000 Histocompatibility Testing.

K1.100 HLA-A,-B,-C,-DR and -DQ typing of all available first degree relatives should be done to establish inheritance of haplotypes.

K1.120 HLA typing for HLA identical siblings (and other first degree relatives) must include adequate testing to definitely establish HLA identity. Molecular HLA typing or augmented testing (e.g., MLC, T cell precursor frequency) should be performed as appropriate for the transplant protocol and optimal donor selection.

K1.130 HLA typing for potential donors who are not first degree relatives must include molecular typing for Class II alleles at a level that is appropriate for the transplant protocol and optimal donor selection. Augmented testing (e.g., molecular typing for Class I HLA, bidirectional MLC, T cell precursor frequency) should be performed as appropriate for the transplant protocol and optimal donor selection.

K2.000 Forward and reverse lymphocytotoxicity and granulocytotoxicity crossmatch tests (patient serum, donor cells and donor serum, patient cells) may be advisable.

K3.000 When the patient has aplastic anemia, every effort should be made to complete tests as rapidly as possible to minimize the number of pretransplant blood transfusions.

K4.000 Unrelated donors.

K4.100 The donor should give his informed consent before blood is taken for typing and before the donor is placed on a list of donors available to be called.

K4.200 Donor records should be maintained so that donors can be rapidly retrieved according to HLA type.

K4.300 Laboratories should have a mechanism in place for resolving any tissue typing discrepancies that may occur between laboratories.

L – PLATELET AND GRANULOCYTE TRANSFUSION

L1.000 HLA Typing.

L1.100 The patient and members of his immediate family should be typed for HLA-A and B antigens.

L1.200 Typing for HLA-C, D, DR, DQ and DP is not necessary.

L2.000 The donor should give his informed consent before blood is taken for typing and before the donor is placed on a list of donors available to be called.

L2.100 Donor records should be maintained so that donors can be rapidly retrieved according to HLA type.

L3.000 Screening the sera of patients for lymphocytotoxic antibodies at intervals is an appropriate way to detect alloimmunization.

L4.000 Crossmatching.

L4.100 Lymphocytotoxic crossmatches are optional.

L4.200 Crossmatching by techniques which utilize donor platelets or granulocytes as the target cells is preferred.

M – DISEASE ASSOCIATION

M1.000 Complete HLA typing is an appropriate option.

M1.100 Typing may also be limited to all products of a single or limited number of HLA loci.

M2.000 Typing for a Single Antigen (e.g., HLA-B27).

M2.100 Cell controls must be tested on each batch of typing-trays.

M2.110 The control cells must include at least two cells known to express the specified antigen.

M2.120 The control cells must also include two cells for each cross-reacting antigen which might be confused with the specific antigen.

M2.130 The control cells must also include at least two cells lacking the specific and crossreacting antigens.

M2.200 Serum controls must be tested at the time of typing.

M2.210 Serum controls must include a positive and negative control.

M2.220 Serum controls should also include two sera for each antigen which crossreacts with the specified antigen (if available).

M2.300 Sera to define each antigen must meet requirements of Sections E or F as appropriate.

N – PARENTAGE TESTING

N1.000 Parentage testing must be restricted to laboratories whose Director fulfills the general Director qualifications (B1.000) and in addition is qualified by advanced training and/or experience in parentage testing.

N1.100 The competency of the technical staff in relation to parentage testing must be the responsibility of the Director.

N1.200 The laboratory Director and technical staff performing parentage testing must participate in continuing education relative to the field of parentage testing.

N1.300 A qualified individual must be available for legal testimony in the case, as needed.

N2.000 Laboratories utilizing genetic systems in addition to HLA must be able to document expertise and/or accreditation in those systems.

N2.100 An accredited laboratory may engage another laboratory to perform genetic testing for systems not used by the primary laboratory. In that event, the subcontracting laboratory and that portion of the testing for which it bears responsibility must be noted in the report (see N7.000).

N3.000 Subject Identification.

N3.100 Evidence for verifiable means of identification for subjects must be recorded at the time the blood sample is taken.

N3.200 Recommended evidence includes photographs, fingerprints and the number(s) of identification cards displaying the subject's picture (e.g., drivers license).

N3.300 Specimens received from an outside collecting facility must also have a means for positive identification unless this requirement has been waived by mutual consent of the individuals involved.

N3.400 A record must be kept at the testing facility of all identifying information including, but not limited to, name, relationship, race, place and date of collection of sample. Information about each individual must be verified by the signature of that person or the guardian.

N3.500 The date of birth of the child and recent transfusion history (past three months) of each individual to be tested must be recorded.

N4.000 Sample Identification.

N4.100 Each tube must be labeled immediately prior to or following collection of the sample to avoid mix-up of samples.

N4.200 The label must include the full name of the subject, the date and the initials of the blood drawer.

N4.300 The phlebotomist's name must be part of the permanent record.

N4.400 A record of the "chain of custody" of the sample must be maintained.

N5.000 HLA Testing Requirements for Parentage Testing.

N5.100 Each test sample must be plated on two separate trays or tray sets each containing a minimum of one monospecific or two multispecific sera defining each HLA-A and B locus antigen tested. The sera defining a particular specificity should be from different donors. The trays must be read independently.

N6.000 Calculations.

N6.100 Computer assisted analyses must be reviewed, verified and signed by the Supervisor and/or Laboratory Director before issue.

N6.200 The computer program which is utilized for analyses must be documented.

N6.300 If only manual calculations are done, they must be done in duplicate.

N6.400 Gene and haplotype frequencies should have been obtained from examination of populations of adequate size.

N7.000 Reports.

N7.100 Each report must be released only to authorized individuals and must contain:

N7.110 The name of each individual tested and the relationship to the child.

N7.120 The racial origin(s) assigned by the laboratory to the mother and alleged father(s) for the purpose of calculation.

N7.130 The phenotypes established for each individual in each genetic system examined.

N7.140 A statement as to whether or not the alleged father can be excluded. When there is no exclusion, the report must contain:

N7.141 The individual Paternity Index for each genetic system reported.

N7.142 The cumulative Paternity Index.

N7.143 The probability of paternity expressed as a percentage. The prior probability(ies) used to calculate the probability of paternity must be stated.

N7.144 Other mathematical or verbal expressions are optional. If they are included in the report, such expressions should be defined and explained.

N7.150 If the results are inconclusive, an explanation as to the nature of the problem.

N7.160 The signature of the laboratory Director.

P – NUCLEIC ACID ANALYSIS

The nucleic acid analysis standards apply to histocompatibility testing.

P1.000 Restriction Fragment Length Polymorphism (RFLP).

P1.100 Restriction Endonucleases.

P1.110 Enzymes must be stored and utilized under conditions recommended by the manufacturer (i.e. storage temperature, test temperature, buffer) to ensure proper DNA digestion.

P1.120 It should be documented that each lot of enzyme produces human DNA polymorphism of known sizes prior to analysis of results.

P1.130 When DNA is digested for analysis, human DNA which will produce polymorphism of known sizes must also be digested to ensure complete endonuclease digestion.

P1.200 Probes.

P1.210 Each DNA probe utilized should be validated by family studies demonstrating Mendelian inheritance of the polymorphism detected and by extensive population studies.

P1.220 The probe should be used in the form as reported in the scientific literature and as was used to determine the inheritance pattern and population distribution of the polymorphism.

P1.300 DNA Extraction.

P1.310 DNA should be purified by a standard method that has been reported in the scientific literature and validated in the laboratory.

P1.320 If the DNA is not used immediately after purification, suitable methods of storage should be available that would protect the integrity of the material.

P1.330 DNA must be intact and not degraded.

P1.400 Electrophoresis.

P1.410 Size markers of known sequences that give discrete electrophoretic bands that span and flank the entire range of the DNA system being tested must be included in the electrophoretic run. The known human control DNA used to determine that complete endonuclease digestion was achieved, must also be included in each electrophoretic run as a control.

P1.420 Equal amounts (mg/ml) of DNA must be loaded per lane.

P1.430 A photograph of the ethidium bromide pattern resulting from the electrophoretic separation should be kept for each run.

P1.500 Prehybridization, Hybridization, Autoradiography.

P1.510 Prehybridization, hybridization, autoradiography must be carried out under empirically determined conditions of concentration, temperature and salt concentration which are determined by the nature of the probe.

P1.520 Stringency conditions should be selected to minimize the possibility of cross-hybridization.

P1.530 Probes should be labeled by a method appropriate for the probe in use. Nick translation, hexamer priming, end labeling or avidin biotin may be appropriate.

P1.540 Each probe used should give a signal adequate to detect a single copy gene. Whenever possible, locus-specific probes should be used.

P1.550 Re-probing of the same membrane should be performed only after complete stripping of the first probe.

P1.600 Analysis

P1.610 Only autoradiographs or membranes that reveal the appropriate patterns of the human control DNA and size markers should be analyzed.

P1.620 Each autoradiograph or membrane should be read independently by two or more individuals.

P1.630 The laboratory report for each fragment detected **should** specify the probe, restriction endonuclease used, fragment size (k.b.) and the chromosomal location as defined by the International Human Gene Mapping Workshop.

P2.000 Amplification-based Typing

P2.1000 Amplification

P2.1100 Laboratory Design.

Use of physical and/or biochemical barriers to prevent DNA contamination (carry-over) is required. Pre-amplification procedures must be performed in a dedicated work area that excludes amplified DNA that has the potential to serve as a template for amplification in the HLA typing assays (e.g., PCR product, plasmids containing HLA genes). Physical separation and restricted traffic flow is recommended. Use of a static air hood or a Class II biological safety cabinet is recommended.

Biochemical procedures can be used to inactivate amplified products.

P2.1200 Other pre-amplification physical containment. Physical containment must include use of dedicated lab coats, gloves and disposable supplies. Frequent cleaning with dilute acid or bleach and/or UV treatment of work surfaces is recommended.

P2.1300 Equipment and Reagents.

P2.1310 Equipment.

P2.1311 Use of dedicated equipment for pre-amplification procedures is recommended.

P2.1312 Use of dedicated pipettors is required. Positive displacement pipettes or filter-plugged tips are recommended.

P2.1313 Thermal cycling instruments must precisely and reproducibly maintain the appropriate temperature of samples. Accuracy of temperature control for samples should be verified on a regular basis.

P2.1320 Reagents.

P2.1321 All reagents (solutions containing one or multiple components) utilized in the amplification assay must be dispensed in aliquots for single use or reagents can be dispensed in aliquots for multiple use if documented to be free of contamination at each use. When reagents are combined to create a master mix, it is recommended that one critical component (e.g. Mg++) be left out of the aliquot.

P2.1322 Reagents (e.g., chemicals, enzymes) must be stored and utilized under conditions recommended by the manufacturer (i.e., storage temperature, test temperature, buffer, concentration). Reagents used for amplification must not be exposed to post-amplification work areas. The appropriate performance of each lot of reagent must be documented before results using these reagents are reported.

P2.1323 For commercial kits, the source, lot number, expiration date, and storage conditions must be documented. Reagents from different

lots of kits must not be mixed. Each laboratory is responsible for the accuracy of typing. One possible approach for quality control is to test each reagent with a positive and negative control.

P2.1324 Primers must be stored under conditions that maintain specificity and sensitivity.

P2.1325 Methods that utilize two consecutive steps of logarithmic amplification are especially susceptible to errors related to PCR carryover (contamination) and special attention must be paid to containment of amplified products (e.g., physical separation, work flow and enhanced contamination monitoring). Standard 2.1100 applies to all components of the second amplification except template. Addition of the template for the second amplification must be physically separated from the pre-amplification work area and the post-amplification work area. Use of pipettors dedicated to each work area (i.e. first amplification, second amplification and analysis) is required.

P2.1400 Amplification templates

P2.1410 Specimens must be stored under conditions that do not result in artifacts or inhibition of the amplification reaction. Specimens must not be exposed to post-amplification work areas.

P2.1420 Nucleic acids should be prepared by a standard method that has been validated in the laboratory.

P2.1430 DNA or cDNA (from RNA templates) is satisfactory. DNA from any nucleated cells or RNA from any cells expressing the HLA product may be used. If RNA is used, appropriate positive controls for reverse transcription must be included.

P2.1440 Nucleic acids must be prepared and stored in a manner which does not result in artifacts or inhibition of the amplification reaction. The acceptable range for the amount of target must be specified and validated.

P2.1500 Primers.

P2.1510 The specificity and sequence of primers must be defined. The HLA locus and allele(s) must be defined.

P2.1520 Conditions which influence the specificity or quantity of amplified product must be demonstrated to be satisfactory for each set of primers.

P2.1530 Reference material should be used to test and periodically reconfirm the specificity and product quantity of each lot of primers.

P2.1600 Contamination.

P2.1610 Nucleic acid contamination must be monitored. Controls must be tested using the method that is routinely used to detect HLA types.

P2.1611 Negative controls (no nucleic acid) must be included in each amplification assay. Another negative control might include open tubes in the work area.

P2.1612 In order to minimize the detection of minor contaminants and the occurrence of stochastic fluctuation the number of cycles should be set at a level sufficient to detect the target nucleic acid but insufficient to detect small amounts (e.g., <10 molecules) of contaminating template.

P2.1613 Routine wipe tests of pre-amplification work areas must be performed. If amplified product is detected, the area must be cleaned to eliminate the contamination and measures must be taken to prevent future contamination.

P2.1700 Controls.

P2.1710 The quantity of specific amplification products must be monitored (e.g., gel electrophoresis, hybridization).

P2.1720 Criteria for accepting or rejecting an amplification assay must be specified.

P2.1730 If presence of an amplified product is used as the end result, controls must be included to detect amplification failure in every amplification mixture. Amplification specificity must be monitored on a periodic basis.

P2.2000 Amplified Product (Nucleic Acid Targets)

P2.2100 Variation in the amount of amplified product must be monitored (e.g., hybidization with a consensus probe, gel electrophoresis). The acceptable range for the amount of available target must be specified.

P2.3000 Oligonucleotide Probes

P2.3100 HLA locus and allele(s) must be defined for each probe and template combination. Positive or negative probe hybridization must be defined for each probe with all possible combinations of alleles that are recognized by the W.H.O. provided that nucleotide sequences are readily available.

P2.3200 Probes must be stored under conditions which maintain specificity and sensitivity.

P2.3300 Probes must be utilized under empirically determined conditions that achieve the defined specificity. The specificity should be demonstrated and maintained for each lot of probe. Each lot of probes should be tested for specificity and product quantity using reference material under optimized conditions and reconfirmed periodically.

P2.3400 Hybridization must be carried out under empirically determined conditions that achieve the defined specificity.

P2.3500 The specificity of hybridization should be confirmed using positive and negative controls for hybridization with each probe. The controls should be capable of detecting cross-hybridization with closely related sequences.

P2.3600 Reuse of nucleic acids (probes or targets) bound to solid supports should only be undertaken after demonstrating that previous signals are no longer detectable.

P2.3700 Reuse of nucleic acids in solution (probes or targets) should only be undertaken with controls to ensure that the sensitivity and specificity of the assay are unaltered.

P2.3800 Incubators and water baths must be monitored for precise and accurate temperature maintenance every time the assay is performed.

P2.4000 Labeling of nucleic acids and detection

P2.4100 The specificity and sensitivity of the labeling and detection method must be established and reproducible.

P2.4200 The specificity and sensitivity must be maintained for each lot of reagents (e.g., antibodies, probes, indicator molecules).

P2.4300 Enzymes must be stored and utilized under conditions recommended by the manufacturer (i.e., storage temperature, test temperature, buffer, concentration) to ensure correct enzymatic activity. The enzymatic activity of each lot should be confirmed before use.

P2.5000 Analysis

P2.5100 Acceptable limits of signal intensity must be specified for positive and negative results. If these are not achieved, corrective action is required.

P2.5200 The method of assignment of types must be designated.

P2.5300 Two independent interpretations of primary data are recommended.

P2.5400 Reports must designate the type of assay (e.g., PCR/oligonucleotide), indicate the HLA locus, and define each type using W.H.O. nomenclature for alleles.

P2.5500 A permanent record of primary data must be retained for 2 years.

P2.6000 Nucleotide Sequencing.

P2.6100 Sequencing Templates.

Standards in P2.1400 must be followed for preparation of templates.

P2.6110 Templates must have sufficient specificity (e.g., locus or allele-specificity), quantity and quality to provide interpretable primary sequencing data. The method for preparing templates must reliably generate appropriate length sequencing templates that are free of

inhibitors of subsequent reactions (e.g., primer extension) and free of contaminants that cause sequencing artifacts. Methods must ensure that preparation of templates does not alter the accuracy of the final sequence (e.g., mutations created during cloning, preferential amplification).

P2.6120 Reagents used in preparation of templates (e.g., enzymes, biochemicals) must be stored and utilized under conditions recommended by the manufacturer. The appropriate performance of each lot must be documented before results of tests using these reagents are reported.

P2.6200 Methods Utilizing Primer Extension.

P2.6210 The specificity and general knowledge of the target sequence must be defined. The HLA locus and allele(s) must be defined.

P2.6220 Primers must be used under empirically determined conditions that achieve the defined specificity of amplification. The amplification conditions must be demonstrated by the laboratory to achieve defined specificity and must yield adequate quantity of specific product. Each lot of primer should be tested for specificity and product quantity using reference material (e.g. DNA) under routine conditions and reconfirmed periodically.

P2.6230 Conditions for primer extension (e.g., polymerase type, polymerase concentration, primer concentration, concentration of nucleoside triphosphates, concentration of terminators) must be appropriate for the template (e.g., length of sequence, GC content).

P2.6240 The specificity and sensitivity of the labeling and detection methods must be documented (e.g., demonstrating correct signal strength for a control sequence) in the laboratory before results are reported.

P2.6250 Satisfactory performance of each lot of reagent (e.g., nucleotides, enzymes) must be documented before results using these reagents are reported. Reagents must be stored under conditions that maintain optimal performance.

P2.6300 Electrophoresis.

P2.6310 A sequencing standard must be run on every gel. The laboratory must establish scientifically and technically sound criteria for accepting each gel and each lane of a gel.

P2.6320 A permanent record of each electrophoretic run (e.g., electronic file, hard copy) must be retained for at least two years.

P2.6330 Satisfactory performance of each lot of reagents that influence the quality and accuracy of sequencing data of the gel (e.g., acrylamide, buffer and salt concentration) should be documented before results using these reagents are reported. Acceptable electrophoretic conditions (e.g., temperature, voltage, duration) must be established. Conditions should be recorded for each run. Reagents must be stored under conditions that maintain acceptable performance.

P2.6400 Nucleotide assignments

P2.6410 Criteria for acceptance of primary data must be established (e.g., correct assignments for nonpolymorphic positions, certain region of sequence, criteria for peak intensity, baseline fluctuation, signal-to-noise ratio and peak shapes). Validation might include sequencing of representatives of all polymorphic motifs that are frequently encountered in the routine sample population to detect sequence-specific artifacts. Sequencing of both strands of at least one representative of each polymorphic motif is recommended during validation. Established sequence-specific characteristics should be documented and utilized in routine interpretation of data.

P2.6420 Routine sequence assignments should be based on analysis of sequence data from complementary strands of DNA unless it is documented that the sequencing method consistently yields accurate sequence assignments using data from only one strand of DNA. If assignments are routinely based upon data from one strand of DNA, periodic confirmation of complementary strands is recommended. If base assignments are frequently difficult to interpret, routine sequencing of both strands is recommended. If a sequence suggests a novel allele or a rare combination of alleles, the sequences of both strands must be determined.

P2.6430 A scientifically sound and technically sound method must be established for interpretation, acceptance, and/or rejection of sequences from regions which are difficult to resolve (e.g., compression, ends).

P2.6440 Two independent interpretations of the primary data are recommended.

P2.6450 Automated systems and computer programs for nucleotide assignments must be validated prior to use.

P2.6500 Allele Assignments

P2.6510 HLA locus and alleles must be defined for each template/primer combination. Each unknown sequence must be compared with the sequences of all alleles that are recognized by the W.H.O. provided that the nucleotide sequences are readily available (i.e., in a locus-specific alignment in conjunction with the W.H.O. Nomenclature Committee for Factors of the HLA System which appears periodically in the public domain such as *Tissue Antigens,* the ASHI Web Pages or *Human Immunology*. Databases of sequences must be accurate and conform to the most recent compilation of sequences published in conjunction with the W.H.O.

P2.6520 Ambiguous combinations of alleles should be defined for each template/primer combination

P2.6530 Methods must ensure that sequences contributed by amplification primers are not considered in the assignment of alleles.

P2.6540 Two independent assignments of alleles are recommended.

P2.6550 Automated systems and computer programs for allele assignments must be validated prior to use.

P2.6560 Reports must designate the type of assay, HLA locus, and define each type using W.H.O. Nomenclature for alleles. The laboratory must maintain records that define the sequence database utilized to interpret the primary data. This database must be updated periodically. If a determined sequence is ambiguous (i.e. more than one possible interpretation of available data) the report must indicate all possible allelic combinations.

P2.7000 Restriction Fragment Length Polymorphism of Amplified Products

P2.7100 Restriction endonucleases.

P2.7110 HLA locus and allele(s) must be defined for each RFLP type.

P2.7120 Enzymes must be stored and utilized under conditions recommended by the manufacturer (i.e., storage temperature, test temperature, buffer, concentration) to ensure correct enzymatic activity. The appropriate performance of each lot of enzyme must be documented before results using these reagents are reported.

P2.7130 When amplified DNA is digested, controls of amplified DNA which will produce fragments of known sizes must also be digested in parallel to monitor complete digestion.

P2.7200 Electrophoresis.

P2.7210 Size markers of known sequence that produce discrete electrophoretic bands spanning and flanking the entire range of expected fragment sizes must be included in every run.

P2.7220 The amount of DNA/lane must not alter the rate of migration with respect to the migration of controls.

P2.7230 A permanent record (e.g., photograph, image) of each electrophoretic run must be retained as defined in C5.1000.

P2.7240 Amplified DNA should be incubated without restriction enzyme and analyzed by gel electropheresis to monitor marker integrity.

P2.7300 Analysis.

P2.7310 Acceptable limits of signal intensity must be specified for positive and negative results. If these are not achieved, corrective action is required.

P2.7320 Appropriate migration patterns of control DNA and size markers are required.

P2.7330 The method of assignment of HLA types must be designated.

P2.7340 Two independent interpretations of primary data are recommended.

P2.7350 Reports must designate the type of assay (e.g., PCR/RFLP), indicate the HLA locus, and define each HLA type using W.H.O. nomenclature for alleles.

P2.8000 Typing Using Sequence-Specific Amplification

P2.8100 HLA locus and allele(s) must be defined for each primer combination. Positive or negative amplification must be defined for each primer mixture with all possible combinations of alleles that are recognized by the W.H.O. provided that nucleotide sequences are readily available.

P2.8200 Each amplification reaction must include procedures to detect technical failures (e.g., an internal control such as additional primers or templates that produce a product that can be distinguished from the typing product).

P2.8300 In each amplification assay (i.e. set up of amplification mixtures for one or more samples) controls should be used to detect contamination with previously amplified products (e.g., a special primer pair internal to all amplification products or a combination of primers to detect any DNA that could confound the typing result).

P2.8400 Primers must be utilized under empirically determined conditions that achieve the defined specificity for templates used in routine testing. Each set of primers must be tested for amplification specificity and product quantity using reference cells under optimized conditions. The frequency of testing each primer set must ensure that all primer pairs have appropriate sensitivity and specificity of amplification. The specificity and sensitivity must be maintained in heterozygous samples.

P2.8500 The specificity and sensitivity of the detection method must be established and reproducible.

P2.8600 Analysis

P2.8610 Acceptable qualitative limits of signal intensity must be specified for positive and negative results. If these are not achieved, corrective action is required.

P2.8620 The method of assignment of types must be designated.

P2.8630 Two independent interpretations of primary data are recommended.

P2.8640 Reports must designate the type of assay (e.g., SSP), indicate the HLA locus, and define each type using W.H.O. nomenclature for alleles.

P2.8650 A permanent record of primary data must be retained for 2 years.

P2.9000 Other Methods

P2.9100 If alternate methods (e.g., SSCP, heteroduplex, DGGE) are used for HLA typing, established procedures must be defined and must include sufficient controls to ensure accurate assignment of types for every sample. All relevant standards from the above sections should be applied.

P2.9200 Automated systems and computer programs must be validated prior to use and tested routinely for accuracy and reproducibility of manipulations.

Q – FLOW CYTOMETRY

These standards apply to histocompatibility testing and leucocyte phenotyping by flow cytometry.

Q1.000 Instrument Standardization/Calibration.

Q1.100 An optical standard, consisting of latex beads or other uniform particles, shall be run to insure proper focusing and alignment of all lenses in the path for both the exciting light source and signal (light scatter, fluorescence, etc.) detectors.

Q1.110 The optical standard shall be run each time the instrument is turned on and any time maintenance, adjustments or sample problems likely to have altered optical alignment (obstruction of fluidics) occur during operation.

Q1.120 The results of optical focusing/alignment must be recorded in a daily quality control log.

Q1.130 A threshold value for acceptable optical standardization must be established for all relevant signals for each instrument and the focusing procedure repeated until these values are achieved or surpassed.

Q1.140 In the event a particular threshold value cannot be attained, a written protocol for instituting corrective action must be available. This protocol should include appropriate corrective actions including clear guidelines describing when a service call is warranted.

Q1.200 A fluorescent standard for each fluorochrome to be used, shall be run to insure adequate amplification of the fluorescent signal(s) on a day-to-day basis.

Q1.210 This standard may be incorporated in the beads or other particles used for optical standardization or may be a separate bead or fixed cell preparation.

Q1.220 The fluorescent standard must be run each time the instrument is turned on and any time maintenance, adjustments or sample problems likely to have altered the gain or high voltage settings (e.g. obstruction of fluidics) occur during operation.

Q1.230 The results of fluorescent standardization shall be recorded in a daily quality control log.

Q1.240 In the event that acceptable fluorescence separation cannot be attained, a written protocol for instituting corrective action must be available. This protocol should include appropriate corrective action including clear guidelines describing when a service call is warranted.

Q1.300 If performing analyses that require the simultaneous use of two or more fluorochromes, an appropriate procedure must be used to compensate for "spill over" into the other fluorescence detectors.

Q1.400 For laser based instruments, the current input (amps) and laser light output (milliwatts), at the normal operating wavelength measured after the laser is peaked and normal operating power set, must be recorded as part of a daily quality control record.

Q2.000 Flow Cytometric Crossmatch Technique

Q2.100 A multi-color technique is highly recommended. However, if a single color technique is used, the purity of the isolated cell population must be documented and should be of sufficient purity to define the population for analysis.

Q2.110 The binding of human immunoglobulin should be assessed with a fluorochrome labelled (e.g., fluorescein) F(ab')2 anti-human IgG.

Q2.120 Binding of antibody to T cells, B cells and/or monocytes should be positively confirmed with a differently labelled (e.g., phycoerythrin) monoclonal antibody that detects the corresponding cluster designated antigen (e.g., CD3 for T cells, CD19 or CD20 for B cells and CD14 for monocytes).

Q2.130 Multicolor staining of other immunoglobulin classes and target cells may also be justified.

Q2.140 Each laboratory should establish and document the optimum serum/cell ratio i.e., a standard number of cells to a fixed volume of serum.

Q2.200 Controls.

Q2.210 The normal human serum control should be from a non-alloimmunized and otherwise healthy individual and must be screened by flow cytometry to insure lack of reactivity against human lymphocytes.

Q2.220 The positive control should be human serum containing antibodies of the appropriate isotype, specific for the HLA antigens or any other alloantigens deemed to be important for detection in the crossmatch. Positive controls should react with lymphocytes of all humans.

Q2.230 The anti-human immunoglobulin reagent should be titered to determine the dilution with optimal activity (signal to noise ratio). If a multicolor technique is employed, the reagent must not demonstrate crossreactivity with the other immunoglobulin reagents used to mark the cells.

Q2.240 Regardless of the method used for reporting raw data (mean, median, mode channel shifts or quantitative fluorescence measurements), each lab must establish its own threshold for discriminating positive reactions. Any significant change in protocol, reagents or instrumentation requires repeat determination of the positive threshold.

Q2.300 Interpretation

Q2.310 Each laboratory must define the criteria used to define positive and negative crossmatches.

Q3.000 Immunophenotyping By Flow Cytometry

Q3.100 Terminology used must be defined and/or conform to nomenclature recommended/approved by the most recent International Workshop of Differentiation Antigens of Human Leucocytes or other appropriate scientific organizations.

Q3.200 Cell Preparation.

Q3.210 The method used for cell preparation should be documented to yield appropriate preparations of viable cells.

Q3.220 The viability of cell preparations should be recorded and should exceed the laboratory's established minimum standards for each procedure used.

Q3.230 For internal labelling, the method used to allow fluorochrome labelled antibodies to penetrate the cell membrane must be documented to be effective.

Q3.300 Labeling of Specimens.

Q3.310 Specificity controls, consisting of appropriate cell types known to be positive for selected standard antibodies must be run within laboratory-defined intervals sufficiently short to assure the proper performance of reagents.

Q3.320 A negative reagent control(s) shall be run for each test cell preparation. This control should consist of monoclonal antibody(ies) of the same species and subclass and should be prepared/purified in the same way as the monoclonal(s) used for phenotyping.

Q3.330 For indirect labelling, the negative control reagent should be an irrelevant primary antibody, if available, and in all cases, the same secondary antibody(ies) conjugated with the same fluorochrome(s) used in all relevant test combinations.

Q3.340 For direct labelling, the negative control reagent should be an irrelevant antibody conjugated with the same fluorochrome and at the same fluorochrome:protein ratio used in all relevant test combinations.

Q3.350 Whether analyzed directly or fixed prior to analysis, labelled cells must be analyzed within a time period demonstrated by the laboratory to avoid significant loss of any cell subpopulation or total cell numbers. Control samples must be analyzed within the same period after staining as the test samples.

Q3.360 If analysis will be based on a population of cells selected by flow cytometry "gating" on size or density parameters, or selected by depletion or enrichment techniques, control stains must be run for each test individual to detect the presence of contaminating cells in the selected population. (e.g., Monocyte contamination of 'lymphocytes' gated by forward angle or forward angle vs 90° light scatter must be detected with a monocyte specific marker antibody.

Q3.370 Conclusions about abnormal proportions or abnormal numbers of cells bearing particular internal or cell surface markers must only be drawn in comparison with local 'control' data obtained with the same instrument, reagents and techniques.

Q3.380 Determination of percent positives must take into consideration the results of the negative control reagent. However, when clearly defined positive and negative populations are evident in the test sample, it may be appropriate to adjust the threshold based on the test sample.

Q3.400 Reagents

Q3.410 The specificity of monoclonal antibodies shall be verified by published and/or manufacturer's documentation and whenever possible verified locally through tests with appropriate control cells prepared and tested by the same method employed in the laboratory's test sample analysis.

Q3.420 The quantities of reagents used for each test sample must be determined by the manufacturers or from published data and whenever possible should be verified locally by appropriate titration procedures.

Q3.430 Reagents must be stored according to manufacturers' instructions or according to conditions verified to maintain stability by documented local tests.

Q3.440 Monoclonal antibodies which have been reconstituted from lyophilized powder form for storage at 4°C should be centrifuged according to the manufacturer's instructions or locally documented procedures to remove microaggregates prior to use in preparation of working stains.

Q4.000 HLA Typing By Flow Cytometry (e.g., HLA B27)

Q4.100 Terminology used must be defined and/or conform to nomenclature recommended/approved by the most recent W.H.O. nomenclature committee meeting.

Q4.200 Cell Preparation.

Q4.210 The method used for cell preparation should be documented to yield appropriate preparations of viable cells.

Q4.220 The viability of cell preparations should be recorded and should exceed the laboratory's established minimum standards for each procedure used.

Q4.2300 Labelling of specimens.

Q4.2310 A negative reagent control(s) shall be run for each test cell preparation. This control should consist of monoclonal antibody(ies) of the same species and subclass and should be prepared/purified in the same way as the monoclonal(s) used for phenotyping. Negative reagent controls should consist of:

Q4.2311 For indirect labelling, an irrelevant primary antibody, if available, and in all cases, the same secondary antibody(ies) conjugated with the same fluorochrome(s) used in all relevant test combinations.

Q4.2312 For direct labelling, an irrelevant antibody conjugated with the same fluorochrome and at the same fluorochrome: protein ratio used in all relevant test combinations.

Q4.2320 Whether analyzed directly or fixed prior to analysis, labelled cells must be analyzed within a time period demonstrated by the laboratory to avoid significant change in test results. Control samples must be analyzed within the same period after staining as the test samples.

Q4.3000 Reagents.

Q4.3100 The specificity of monoclonal antibodies shall be verified through tests with appropriate control cells prepared and tested by the same method employed in the laboratory's test sample.

Q4.3200 Cell controls must be tested for each batch of monoclonal antibodies received.

Q4.3210 The control cells must include at least five cells known to express the specified antigen.

Q4.3220 The control cells must also include two cells for each cross-reacting antigen which might be confused with the specific antigen.

Q4.3230 The control cells must also include at least two cells lacking the specific and crossreacting antigens.

Q4.3300 The quantities of reagents used for each test sample must be determined by the manufacturers or from published data and whenever possible should be verified locally by appropriate titration procedures.

Q4.3400 Reagents must be stored according to manufacturer's instructions or according to conditions verified to maintain stability by documented local tests.

Q4.3500 Monoclonal antibodies which have been reconstituted from lyophilized powder form for storage at 4 degrees centigrade should be centrifuged according to the manufacturer's instructions or locally documented procedures to remove microaggregates prior to use in preparation of working stains.

Q4.3600 A single monoclonal antibody may be used to define an antigen provided its monospecificity has been sufficiently verified by local testing.

Q4.3700 Minimum reactivity for assignment of a positive reaction must be established by the laboratory.

Q4.3800 If the monoclonal antibody(ies) is (are) known or found to react with antigens other than the one specified, a written protocol must explain how its presence or absence is finally determined.

R – ENZYME-LINKED IMMUNO SORBENT ASSAY (ELISA)

R1.000 Instrument Standardization/Calibration.

R1.100 The ELISA reader.

R1.110 The light source and filter must produce the intensity and wavelength of light required for the test system.

R1.120 Precise movement of the plate must be verified and recorded.

R1.130 Periodic calibration must be performed according to the instrument manufacturer's instructions and must be documented.

R1.200 Assays must be performed with calibrated dispensing instruments. Calibration must be routinely performed routinely and must be documented.

R1.300 Microplate washer performance must be checked monthly and acceptable performance documented.

R2.000 ELISA Technique.

R2.100 If commercial kits are used, the manufacturer's instructions must be followed unless the laboratory has performed and documented testing to support a deviation in technique or analysis.

R2.200 Reagents must be stored at the temperature and for no longer than the duration specified by the manufacturer.

R2.300 Each assay must contain a positive control, a negative control and reagent controls. The dilution of reagents and test specimens must be documented.

R2.400 Sample identity and proper plate orientation must be maintained throughout the procedure.

R2.500 The lot numbers and optical density values of the reference reagents and the controls must be recorded for each assay. These values must fall within acceptable limits for the assay to be valid.

R2.600 The volume and number of washes must be recorded for each assay.

R2.700 New lots of reagents must be validated by side-by-side testing with a lot known to give acceptable performance or by testing with test specimens of known reactivity.

HLA Alleles and Equivalent Serological Types

HLA-A Locus Alleles and Equivalent Serological Types

A*0101	A1
A*0102	A1
A*0103	Not defined
A*0104N	Null
A*0201	A2
A*0202	A2
A*0203	A203
A*0204	A2
A*0205	A2
A*0206	A2
A*0207	A2
A*0208	A2
A*0209	A2
A*0210	A210
A*0211	A2
A*0212	A2
A*0213	A2
A*0214	A2
A*0215N	Null
A*0216	A2
A*0217	A2
A*0218	A2
A*0219	Not defined
A*0220	A2
A*0221	A2
A*0222	A2
A*0224	A2
A*0225	A2
A*0226	Not defined
A*0227	Not defined
A*0228	Not defined
A*0229	A2
A*0230	Not defined
A*0301	A3
A*0302	A3
A*0303N	Null
A*0304	A3
A*1101	A11
A*1102	A11
A*1103	A11
A*1104#	A11
A*1105#	A11
A*2301	A23 (9)
A*2402	A24 (9)
A*2402102L	Low A24
A*2403	A2403
A*2404	A24 (9)
A*2405	A24 (9)
A*2406	A24 (9)
A*2407	A24 (9)
A*2408	A24 (9)
A*2409N	Null
A*2410	A9
A*2411N	Null
A*2413	A24 (9)
A*2414	A24(9)
A*2415	Not defined
A*2416#	Not defined
A*2417	Not defined
A*2418#	Not defined
A*2419#	Not defined
A*2501	A25 (10)
A*2502	A25 (10)
A*2601	A26 (10)
A*2602	A26 (10)
A*2603	A26 (10)
A*2604	A26 (10)
A*2605	A26 (10)
A*2606	A26 (10)
A*2607	A26 (10)
A*2608	A26 (10)
A*2609	Not defined
A*2610#	A10
A*2611N	Null
A*2612	Not defined
A*2901	A29 (19)
A*2902	A29 (19)
A*2903	Not defined
A*2904	Not defined
A*3001	A30 (19)
A*3002	A30 (19)
A*3003	A30 (19)
A*3004	A30 (19)
A*3006	Not defined
A*3007	Not defined
A*3101	A31 (19)
A*3102	Not defined
A*3103	Not defined
A*3104	A31 (19)
A*3201	A32 (19)
A*3202	A32 (19)
A*3203	Not defined
A*3301	A33 (19)
A*3303	A33 (19)
A*3304	Not defined
A*3401	A34 (10)
A*3402	A34 (10)
A*3601	A36
A*4301	A43
A*6601	A66 (10)
A*6602	A66 (10)
A*6603	A10
A*6801	A68 (28)
A*6802	A68 (28)
A*6803	A28
A*6804	Not defined
A*6805	Not defined
A*6806	Not defined
A*6807	Not defined
A*6808	A68 (28)
A*6809	Not defined
A*6901	A69 (28)
A*7401	A74 (19)
A*7402	A74 (19)
A*7403	A19
A*8001	A80

For description of serological pattern, see Table 9 of Schreuder et al., The HLA dictionary 1999: a summary of HLA-A, -B, -C, -DRB1/3/4/5, -DQB1 alleles and their association with serologically defined HLA-A, -B, -DR and -DQ antigens. Tissue Antigens 1999; 54:409-437. Reprinted with permission.

HLA-B Locus Alleles and Equivalent Serological Types

B*0702	B7
B*0703#	B703
B*0704	B7
B*0705	B7
B*0706	B7
B*0707	B7
B*0708#	Not defined
B*0709	B7
B*0710	Not defined
B*0711	B7
B*0712	Not defined
B*0713	Not defined
B*0801	B8
B*0802#	B8
B*0803	B8
B*0804#	Not defined
B*0805	Not defined
B*0806	B8
B*1301	B13
B*1302	B13
B*1303#	Not defined
B*1304#	Not defined
B*1401	B64 (14)
B*1402	B65 (14)
B*1403	Not defined
B*1404	Not defined
B*1405	Not defined
B*1501	B62 (15)
B*1502	B75 (15)
B*1503	B72 (70)
B*1504	B62 (15)
B*1505	B62 (15)
B*1506	B62 (15)
B*1507	B62 (15)
B*1508	B75 (15)
B*1509	B70
B*1510	B71 (70)
B*1511	B75 (15)
B*1512	B76 (15)
B*1513	B77 (15)
B*1514	B76 (15)
B*1515	B62 (15)
B*1516	B63 (15)
B*1517	B63 (15)
B*1518	B71 (70)
B*1519	B76 (15)
B*1520	B62 (15)
B*1521	B75 (15)
B*1522	B35
B*1523#	Not defined
B*1524#	B62 (15)
B*1525	B62 (15)
B*1526N	Null
B*1527	B62 (15)
B*1528	B15
B*1529	B15
B*1530	B62 (15)
B*1531	B75 (15)
B*1532	B62 (15)
B*1533	B15
B*1534	B15
B*1535	B15
B*1536	Not defined
B*1537#	Not defined
B*1538#	Not defined
B*1539	Not defined
B*1540	Not defined
B*1542	Not defined
B*1543	Not defined
B*1544	Not defined
B*1545	B62 (15)
B*1546	B72 (70)
B*1547	Not defined
B*1548	B62 (15)
B*1549	Not defined
B*1801	B18
B*1802	B18
B*1803	B18
B*1804	Not defined
B*1805	B18
B*1806#	B18
B*1807	Not defined
B*2701	B27
B*2702	B27
B*2703	B27
B*2704	B27
B*2705	B27
B*2706	B27
B*2707	B27
B*2708#	B2708
B*2709	B27
B*2710	B27
B*2711#	B27
B*2712#	Not defined
B*2713	B27
B*2714	Not defined
B*2715#	Not defined
B*3501	B35
B*3502	B35
B*3503	B35
B*3504	B35
B*3505	B35
B*3506	B35
B*3507	B35
B*3508	B35
B*3509	B35
B*3510	Not defined
B*3511	B35
B*3512	B35
B*3513	B35
B*3514	B35
B*3515#	B35
B*3516	Not defined
B*3517	B35
B*3518	B35
B*3519	B35
B*3520	B35
B*3521	Not defined
B*3522	Not defined
B*3523	Not defined
B*3524	Not defined
B*3525	Not defined
B*3526	Not defined
B*3527	B35
B*3701	B37
B*3702#	Not defined
B*3801	B38 (16)
B*3802	B38 (16)
B*3803#	B16
B*3901	B3901
B*3902	B3902
B*3903	B39 (16)
B*3904	B39 (16)
B*3905#	B16
B*3906	B39 (16)
B*3907	Not defined
B*3908	B39 (16)
B*3909	B39 (16)
B*3910	B39 (16)
B*3911	Not defined
B*3912	B39 (16)
B*3913	B39 (16)
B*3914	Not defined
B*3915	Not defined
B*3916	Not defined
B*4001	B60 (40)
B*4002	B61 (40)
B*4003	B40
B*4004	B40
B*4005	B4005
B*4006	B61 (40)
B*4007	Not defined
B*4008#	Not defined
B*4009	B61 (40)
B*4010#	B60 (40)
B*4011	B40
B*4012#	Not defined
B*4013	Not defined
B*4014	Not defined

B*4015	Not defined	B*4803	Not defined	B*5502	B55 (22)
B*4016	B61	B*4804	Not defined	B*5503#	Not defined
B*4018	Not defined	B*4805	B48	B*5504	B55 (22)
B*4019	Not defined	B*4901	B49 (21)	B*5505	B22
B*4020	Not defined	B*5001	B50 (21)	B*5507	B54 (22)
B*4101	B41	B*5002#	B45 (12)	B*5508	Not defined
B*4102	B41	B*5101	B51 (5)	B*5601	B56 (22)
B*4103	Not defined	B*5102	B5102	B*5602	B56 (22)
B*4201	B42	B*5103	B5103	B*5603#	B22
B*4202	B42	B*5104	B51 (5)	B*5604#	B56 (22)
B*4402	B44 (12)	B*5105	B51 (5)	B*5605	Not defined
B*4403	B44 (12)	B*5106#	B5	B*5701	B57 (17)
B*4404	B44 (12)	B*5107	B51 (5)	B*5702	B57 (17)
B*4405	B44 (12)	B*5108	B51 (5)	B*5703	B57 (17)
B*4406#	B44 (12)	B*5109	B51 (5)	B*5704	B57 (17)
B*4407	B44 (12)	B*5110	Not defined	B*5705	Not defined
B*4408#	B44 (12)	B*5111N	Null	B*5801	B58 (17)
B*4409#	B12	B*5112#	Not defined	B*5802	B58 (17)
B*4410	Not defined	B*5113	Not defined	B*5901	B59
B*4411	Not defined	B*5114	Not defined	B*6701	B67
B*4501	B45 (12)	B*5115	Not defined	B*7301	B73
B*4502	Not defined	B*5116	B52 (5)	B*7801	B78
B*4601	B46	B*5201	B52 (5)	B*7802	B78
B*4701	B47	B*5301	B53	B*7803	Not defined
B*4702#	Not defined	B*5302	Not defined	B*8101	B81
B*4703#	Not defined	B*5303	Not defined	B*8201#	Not defined
B*4801	B48	B*5401	B54 (22)		
B*4802	B48	B*5501	B55 (22)		

For description of serological pattern, see Table 9 of Schreuder et al., The HLA dictionary 1999: a summary of HLA-A, -B, -C, -DRB1/3/4/5, -DQB1 alleles and their association with serologically defined HLA-A, -B, -DR and -DQ antigens. Tissue Antigens 1999; 54:409-437. Reprinted with permission.

HLA-C Locus Alleles and Equivalent Serological Types

Cw*0102	Cw1	Cw*0604	Not defined	Cw*1205	Not defined
Cw*0103	Cw1	Cw*0701	Cw7	Cw*1206	Not defined
Cw*0202	Cw2	Cw*0702	Cw7	Cw*1301	Not defined
Cw*0203	Not defined	Cw*0703	Not defined	Cw*1402	Not defined
Cw*0302	Cw10 (w3)	Cw*0704	Cw7	Cw*1403	Not defined
Cw*0303	Cw9 (w3)	Cw*0705	Not defined	Cw*1404	Not defined
Cw*0304	Cw10 (w3)	Cw*0706	Cw7	Cw*1502	Not defined
Cw*0305	Not defined	Cw*0707	Not defined	Cw*1503	Not defined
Cw*0306	Not defined	Cw*0708	Not defined	Cw*1504	Not defined
Cw*0307	Cw3	Cw*0709	Not defined	Cw*1505	Not defined
Cw*0308	Not defined	Cw*0710	Not defined	Cw*1506	Not defined
Cw*0309	Not defined	Cw*0711	Not defined	Cw*1507	Not defined
Cw*0401	Cw4	Cw*0712	Not defined	Cw*1508	Not defined
Cw*0402	Cw4	Cw*0801	Cw8	Cw*1601	Not defined
Cw*0403	Not defined	Cw*0802	Cw8	Cw*1602	Not defined
Cw*0404	Not defined	Cw*0803	Cw8	Cw*1604	Not defined
Cw*0405	Not defined	Cw*0804	Not defined	Cw*1701	Not defined
Cw*0406	Not defined	Cw*0805	Not defined	Cw*1702	Not defined
Cw*0501	Cw5	Cw*0806	Not defined	Cw*1801	Not defined
Cw*0502	Cw5	Cw*1202	Not defined	Cw*1802	Not defined
Cw*0602	Cw6	Cw*1203	Not defined		
Cw*0603	Not defined	Cw*1204	Not defined		

Schreuder et al., The HLA dictionary 1999: a summary of HLA-A, -B, -C, -DRB1/3/4/5, -DQB1 alleles and their association with serologically defined HLA-A, -B, -DR and -DQ antigens. Tissue Antigens 1999; 54:409-437. Reprinted with permission.

HLA-DR Locus Alleles and Equivalent Serological Types

DRB1*0101	DR1
DRB1*0102	DR1
DRB1*0103	DR103
DRB1*0104	DR1
DRB1*0105	Not defined
DRB1*0106	Not defined
DRB1*0301	DR17 (3)
DRB1*0302	DR18 (3)
DRB1*0303	DR18 (3)
DRB1*0304	DR17 (3)
DRB1*0305	DR17 (3)
DRB1*0306	DR3
DRB1*0307	Not defined
DRB1*0308	Not defined
DRB1*0309	Not defined
DRB1*0310	Not defined
DRB1*0311	Not defined
DRB1*0312	Not defined
DRB1*0313	Not defined
DRB1*0401	DR4
DRB1*0402	DR4
DRB1*0403	DR4
DRB1*0404	DR4
DRB1*0405	DR4
DRB1*0406	DR4
DRB1*0407	DR4
DRB1*0408	DR4
DRB1*0409	DR4
DRB1*0410	DR4
DRB1*0411	DR4
DRB1*0412	Not defined
DRB1*0413	DR4
DRB1*0414	DR4
DRB1*0415#	DR4
DRB1*0416	DR4
DRB1*0417	DR4
DRB1*0418	Not defined
DRB1*0419	DR4
DRB1*0420	DR4
DRB1*0421	DR4
DRB1*0422#	DR4
DRB1*0423	DR4
DRB1*0424	DR4
DRB1*0425#	DR4
DRB1*0426	DR4
DRB1*0427	Not defined
DRB1*0428	DR4
DRB1*0429	DR4
DRB1*0430	Not defined
DRB1*0431	Not defined
DRB1*0432	Not defined
DRB1*0701	DR7
DRB1*0703	DR7
DRB1*0704	Not defined
DRB1*0801	DR8
DRB1*0802	DR8
DRB1*0803	DR8
DRB1*0804	DR8
DRB1*0805	DR8
DRB1*0806	DR8
DRB1*0807	DR8
DRB1*0808	Not defined
DRB1*0809#	DR8
DRB1*0810	DR8
DRB1*0811	DR8
DRB1*0812	DR8
DRB1*0813	Not defined
DRB1*0814	DR8
DRB1*0815	Not defined
DRB1*0816	DR8
DRB1*0817	DR8
DRB1*0818	Not defined
DRB1*0819	Not defined
DRB1*0820	Not defined
DRB1*0821	Not defined
DRB1*0901	DR9
DRB1*1001	DR10
DRB1*1101	DR11 (5)
DRB1*1102	DR11 (5)
DRB1*1103	DR11 (5)
DRB1*1104	DR11 (5)
DRB1*1105	DR11 (5)
DRB1*1106	DR11 (5)
DRB1*1107#	Not defined
DRB1*1108	DR11 (5)
DRB1*1109	DR11 (5)
DRB1*1110	Not defined
DRB1*1111#	Not defined
DRB1*1112	Not defined
DRB1*1113#	DR11 (5)
DRB1*1114	DR11 (5)
DRB1*1115	Not defined
DRB1*1116#	Not defined
DRB1*1117	Not defined
DRB1*1118	Not defined
DRB1*1119	Not defined
DRB1*1120#	DR11 (5)
DRB1*1121	DR11 (5)
DRB1*1122	Not defined
DRB1*1123	DR11 (5)
DRB1*1124	Not defined
DRB1*1125	DR11 (5)
DRB1*1126	DR11 (5)
DRB1*1127	DR11 (5)
DRB1*1128	Not defined
DRB1*1129	DR11 (5)
DRB1*1130	Not defined
DRB1*1131	Not defined
DRB1*1132	Not defined
DRB1*1133	Not defined
DRB1*1134	Not defined
DRB1*1135	Not defined
DRB1*1201	DR12 (5)
DRB1*1202	DR12 (5)
DRB1*1203	DR12 (5)
DRB1*1204#	Not defined
DRB1*1205	DR12 (5)
DRB1*1206	DR12 (5)
DRB1*1301	DR13 (6)
DRB1*1302	DR13 (6)
DRB1*1303	DR13 (6)
DRB1*1304	DR13 (6)
DRB1*1305	DR13 (6)
DRB1*1306	DR13 (6)
DRB1*1307	DR13 (6)
DRB1*1308	DR13 (6)
DRB1*1309	Not defined
DRB1*1310	DR13 (6)
DRB1*1311#	DR13 (6)
DRB1*1312#	DR6
DRB1*1313	Not defined
DRB1*1314	DR13 (6)
DRB1*1315	Not defined
DRB1*1316	DR13 (6)
DRB1*1317#	DR13 (6)
DRB1*1318	DR13 (6)
DRB1*1319#	Not defined
DRB1*1320	DR13 (6)
DRB1*1321	Not defined
DRB1*1322	Not defined
DRB1*1323	Not defined
DRB1*1324	Not defined
DRB1*1325	Not defined
DRB1*1326#	Not defined
DRB1*1327	DR13 (6)
DRB1*1328	Not defined
DRB1*1329	DR6
DRB1*1330	Not defined
DRB1*1331	Not defined
DRB1*1332	Not defined
DRB1*1333	Not defined
DRB1*1334	Not defined
DRB1*1401	DR14 (6)
DRB1*1402#	DR14 (6)
DRB1*1403	DR1403
DRB1*1404	DR1404
DRB1*1405	DR14 (6)
DRB1*1406#	DR14 (6)
DRB1*1407	DR14 (6)

DRB1*1408	Not defined
DRB1*1409	Not defined
DRB1*1410	Not defined
DRB1*1411#	DR14 (6)
DRB1*1412	DR14 (6)
DRB1*1413	DR14 (6)
DRB1*1414	DR14 (6)
DRB1*1415#	DR8
DRB1*1416#	DR6
DRB1*1417#	DR6
DRB1*1418	DR6
DRB1*1419#	DR14 (6)
DRB1*1420#	DR14 (6)
DRB1*1421#	DR6
DRB1*1422#	Not defined
DRB1*1423	Not defined
DRB1*1424	Not defined
DRB1*1425	Not defined
DRB1*1426	DR14 (6)
DRB1*1427	DR14 (6)
DRB1*1428	Not defined
DRB1*1429	DR14 (6)
DRB1*1430	Not defined
DRB1*1431	Not defined
DRB1*1432	Not defined
DRB1*1433	Not defined
DRB1*1501	DR15 (2)
DRB1*1502	DR15 (2)
DRB1*1503	DR15 (2)
DRB1*1504	DR15 (2)
DRB1*1505	DR15 (2)
DRB1*1506	DR15 (2)
DRB1*1507	Not defined
DRB1*1508	DR2
DRB1*1601	DR16 (2)
DRB1*1602	DR16 (2)
DRB1*1603	DR2
DRB1*1604	DR16 (2)
DRB1*1605	DR2
DRB1*1607	Not defined
DRB1*1608	Not defined
DRB3*0101	DR52
DRB3*0102	Not defined
DRB3*0103	Not defined
DRB3*0104	Not defined
DRB3*0105	Not defined
DRB3*0201	DR52
DRB3*0202	DR52
DRB3*0203	DR52
DRB3*0204	Not defined
DRB3*0205	Not defined
DRB3*0206	Not defined
DRB3*0207	DR52
DRB3*0208	DR52
DRB3*0301	DR52
DRB3*0302	DR52
DRB3*0303	Not defined
DRB4*0101	DR53
DRB4*0102	DR53
DRB4*0103	DR53
DRB4*0103102N	Null
DRB4*0104	Not defined
DRB4*0105	DR53
DRB4*0201N	Null
DRB4*0301N	Null
DRB5*0101	DR51
DRB5*0102	DR51
DRB5*0103	Not defined
DRB5*0104	Not defined
DRB5*0105	Not defined
DRB5*0106	Not defined
DRB5*0107	DR51
DRB5*0108N	Null
DRB5*0109	Not defined
DRB5*0110N	Null
DRB5*0202	DR51
DRB5*0203	Not defined
DRB5*0204	Not defined

For description of serological pattern, see Table 10 of Schreuder et al., The HLA dictionary 1999: a summary of HLA-A, -B, -C, -DRB1/3/4/5, -DQB1 alleles and their association with serologically defined HLA-A, -B, -DR and -DQ antigens. Tissue Antigens 1999; 54:409-437. Reprinted with permission.

HLA-DQ Locus Alleles and Equivalent Serological Types

DQB1*0201	DQ2	DQB1*0309	Not defined	DQB1*0605	DQ6 (1)
DQB1*0202	DQ2	DQB1*0401	DQ4	DQB1*0606	Not defined
DQB1*0203	DQ2	DQB1*0402	DQ4	DQB1*0607	Not defined
DQB1*0301	DQ7 (3)	DQB1*0501	DQ5 (1)	DQB1*0608	Not defined
DQB1*0302	DQ8 (3)	DQB1*0502	DQ5 (1)	DQB1*0609	DQ6 (1)
DQB1*0303	DQ9 (3)	DQB1*0503	DQ5 (1)	DQB1*0610	Not defined
DQB1*0304	DQ7 (3)	DQB1*0504	DQ5 (1)	DQB1*0611	DQ1
DQB1*0305	DQ8 (3)	DQB1*0601	DQ6 (1)	DQB1*0612	DQ1
DQB1*0306	DQ3	DQB1*0602	DQ6 (1)	DQB1*0613	Not defined
DQB1*0307	Not defined	DQB1*0603	DQ6 (1)	DQB1*0614	DQ6 (1)
DQB1*0308	Not defined	DQB1*0604	DQ6 (1)	DQB1*0615	Not defined

Schreuder et al., The HLA dictionary 1999: a summary of HLA-A, -B, -C, -DRB1/3/4/5, -DQB1 alleles and their association with serologically defined HLA-A, -B, -DR and -DQ antigens. Tissue Antigens 1999; 54:409-437. Reprinted with permission.

AMERICAN SOCIETY FOR HISTOCOMPATIBILITY AND IMMUNOGENETICS

ASHI Laboratory Manual

Fourth Edition

Volume II

Copyright © 2000. American Society for Histocompatibility and Immunogenetics. All rights reserved.

Editors

Amy B. Hahn, PhD, dip.ABHI
Geoffrey A. Land, PhD, HCLD
Rosemarie M. Strothman

Section Editors

Serology:

Cynthia E. Blanck, PhD
Donna L. Phelan, BA, CHS, MT(HEW)

Cellular:

Patrick W. Adams, MS, CHS
Lois E. Regen, MS, BA, CHS

Molecular Testing:

Debra Kukuruga, PhD, dip.ABHI
Harriet Noreen, CHS

Flow Cytometry:

Joan E. Holcomb, MS, CHS
Lauralynn K. Lebeck, PhD, MS, dip.ABHI

Quality Assurance:

Deborah O. Crowe, PhD, dip.ABHI

ASHI
Laboratory Manual
4th Edition

Table of Contents

VOLUME II: Molecular Testing, Flow Cytometry, and Quality Assurance

V. MOLECULAR TESTING

A. DNA ISOLATION

HLA Class I and Class II DNA Extraction Methods V.A.1.1
Carol Kosman

B. DESIGN AND LABELING OF PRIMERS AND PROBES
Deborah O. Crowe

Overview V.B.1.1

Use of Primers With SSP Methods V.B.1.3

Preparation of Primer and Probe Solutions V.B.1.3

Target DNA V.B.1.4

Nucleic Acid Labeling V.B.1.4

Incorporation of Modified Nucleotide Triphosphates (NTP or dNTP) V.B.1.5

Digoxigenin Labeling for SSOP V.B.1.6

Labeling of Double Stranded DNA (Nick and Random) V.B.1.8

Non-isotopic Labeling. V.B.1.9

Detection Methods V.B.1.11

C. HLA TYPING

PCR-SSP Typing of Class I and Class II Alleles. V.C.1.1
Mike Bunce and Ken Welsh

PCR-SSOP, Class I and Class II (DRB1) V.C.2.1
Derek Middleton

HLA-DPA1 and -DPB1 Typing Using the Polymerase Chain Reaction and Non-Radioactive Sequence-Specific Oligonucleotide Probes. V.C.3.1
Lori L. Steiner, Priscilla V. Moonsamy, Teodorica L. Bugawan and Ann B. Begovich

Analysis of HLA-Class II DRB1 Alleles Using PCR-RFLP V.C.4.1
Julio C. Delgado, Doreen E. Sese, Edgar L. Milford, and Edmond J. Yunis

Enzyme-Linked DNA Oligotyping Performed in Microtiter Plates (ELDOT) V.C.5.1
Aloke Mohinen and Marcelo Fernandez-Vina

Commercial Vendors of Kits for Molecular Typing V.C.6.1
Brian F. Duffy

Analysis of HLA Class I Alleles via Direct Sequencing of PCR Products V.C.7.1
Jin Wu, Sue Bassinger, Barbara B. Griffith, and Thomas M. Williams

HLA-DR Sequence-Based Typing V.C.8.1
Lee Ann Baxter-Lowe

D. POST TRANSPLANT MONITORING

Stem Cell Engraftment Analysis Using PCR Amplification of VNTR/STR Loci V.D.1.1
Anajane G. Smith and Chris McFarland

E. MISCELLANEOUS

Analysis of HLA Alleles Using the TaqMan Method. V.E.1.1
William A. Rudert and Massimo Trucco

Quantitation of Cytokines by Competitive PCR. V.E.2.1
Patrizia Luppi and Massimo Trucco

MHC Microsatellite Analysis V.E.3.1
Maureen P. Martin and Mary Carrington

VI. FLOW CYTOMETRY

A. BASIC PRINCIPLES

Basic Principles and Quality Assurance of Immunofluorescence and Flow Cytometry. VI.A.1.1
Mary S. Leffell and Robert A. Bray

B. FLOW CYTOMETRY CROSSMATCH AND ANTIBODY DETECTION

Cell Based, Flow Cytometric Detection of Panel Reactive Antibody (FC-PRA): Set Up, Acquisition of Data & Analysis VI.B.1.1
Lisa Wilmoth-Hosey, Pam Chapman, Joan Holcomb, and Robert A. Bray

Antibody Detection by Flow Cytometry Using Antigen Coated Beads VI.B.2.1
Lisa Wilmoth-Hosey and Robert A. Bray

Antibody Identification by Flow Cytometry Using HLA Class I or Class II Antigen Coated Specificity Beads. VI.B.3.1
Lisa Wilmoth-Hosey and Robert A. Bray

Flow Cytometric T and B Cell Crossmatching VI.B.4.1
Charles Hamrick and Lauralynn Lebeck

C. CELLULAR TYPING

Phenotyping by Immunofluorescence VI.C.1.1
Mary L. Duenzl, Linda Stempora, Robert A. Bray

HLA-B27 Typing By Flow Cytometry VI.C.2.1
Anne M. Ward

CD34 Enumeration VI.C.3.1
M. Fran Keller and Lauralynn K. Lebeck

D. MISCELLANEOUS

Flow Cytometric Detection of Intracellular Cytokine Production VI.D.1.1
Howard M. Gebel, John W. Ortegel, and Anat R. Tambur

Quantitative Plasma OKT3 Levels VI.D.2.1
Leah N. Hartung and Carl T. Wittwer

VII. QUALITY ASSURANCE

Note: This section is being repeated for the convenience of the user.

A. THE QUALITY ASSURANCE / IMPROVEMENT PROGRAM
Deborah O. Crowe

Overview VII.A.1.1
Manual VII.A.1.2
Forms VII.A.1.5
The Quality Assurance Report VII.A.1.5
Process Improvement VII.A.1.6
Benefits of a Quality Assurance Program VII.A.1.6

B. QUALITY ASSURANCE OF INFORMATION / DATA IN THE LABORATORY
Lori Dombrausky-Osowski

New Test Validation VII.B.1.1
Patient Test Management VII.B.1.2
Computer Validation VII.B.1.4
Laboratory Data Maintenance VII.B.1.4

C. FACILITIES AND ENVIRONMENT
Geoffrey A. Land

Physical Facilities VII.C.1.1
Biologic and Chemical Hazards VII.C.1.6
Radiation Hazards VII.C.1.13

D. QUALITY CONTROL PROGRAM
Anthony L. Roggero and Deborah O. Crowe

Principle/Purpose VII.D.1.1
Proficiency Testing VII.D.1.1
Reagent Quality Control VII.D.1.1
Complement Quality Control VII.D.1.3
Anti-Human Globulin Quality Control VII.D.1.3
Primer Quality Control for DNA Typing VII.D.1.4
Probe Quality Control for DNA Typing VII.D.1.5
Titration of FITC-Anti-Human IgG for Flow Crossmatching VII.D.1.6
Equipment Maintenance VII.D.1.6

Synthesis of Rare DRB1 Allele Sequences for Quality Control of SSOP VII.D.2.1
Debra D. Hiraki, Shalini Krishnaswamy, Carl F. Grumet

Quality Control for DNA Contamination VII.D.3.1
Jeffrey M. McCormack

E. REGULATORY AGENCIES

The Joint Commission of Healthcare Organizations VII.E.1
Anne Belanger

ASHI – The HCFA Perspective VII.E.2
Sandra Pearson and Esther-Marie Carmichael

VIII. APPENDICES

A. CONTRIBUTORS

B. STANDARDS

C. HLA ALLELES AND EQUIVALENT SEROLOGICAL TYPES

HLA Class I and Class II DNA Extraction Methods

Carol Kosman
13th IHW Organizing Committee for DNA Based Typing

Introduction

Methods for DNA extraction from a variety of body fluids and/or tissues can be separated into a few general categories:

1. Those which require a pre-lysing step in which red blood cells are lysed and removed in a pellet; nuclei are freed from PCR-inhibiting heme proteins and other cellular constituents via salt buffers and pelleted
2. Those with the addition of strong cationic detergents (SDS, DTAB) to facilitate nuclear lysis
3. DNA is separated from protein either via high salt, alcohol, detergent, or organic solvents
4. No pre-lysing step is needed, whole blood is directly loaded onto a spin column where cells are captured on a matrix and lysed, then DNA is selectively purified and released

The requirement for an additional DNA precipitation step via ethanol also varies from protocol to protocol.
Below is a summary of the methods described:

Method Procedure	Chelex	Sucrose/Triton (S/T)	Salting Out and Variation	Phenol/Chloroform and Variation	DTAB/CTAB Variation	Affinity Column from Commercial Vendors
RBC Lysis	Pellet RBC	Sucrose/Triton X	Low salt	Low salt;NH4Cl	DTAB/Heat	Chemicals
Nuclear Lysis/ Protein Clearing	Chelate metals High heat	Tween-20 Proteinase K	High salt; SDS/Proteinase K	High salt; SDS/Proteinase K	Chloroform	Detergent; Proteinase K
DNA/Protein separation	Heat Pellet proteins	Heat	High salt; Heat	Phenol/Chloroform Isoamyl Alcohol	CTAB/Low salt	EtOH Silica matrix via osmotic selection
DNA precipitation	No purification	No purification	EtOH	EtOH	EtOH	Elution from matrix via solubilization

Protocols

I. DNA Extraction Methods

DNA can be isolated from a variety of sample sources including anti-coagulated whole blood, clotted whole blood, buffy coat cells, frozen white blood cell pellets, cryopreserved lymphocytes, lymphoblastoid cell lines, and buccal epithelial cells. In general, for situations requiring the testing of many samples, anti-coagulated whole blood is the most convenient source of cells. A major advantage of DNA typing methods is that good quality DNA can be isolated from samples as long as 2 weeks after the initial blood draw, although it is recommended that samples be processed within 2 to 3 days of draw. If whole blood samples are not processed immediately, it is recommended that they be held at room temperature in their original, sterile containers. If processing is delayed more than 1 week, it is recommended that the sample be distributed in small (0.5-1 ml) aliquots and frozen at -20°C to -70°C. Frozen whole blood samples can be stored for at least 1 year without compromising the quality or quantity of the DNA isolated. If it is desirable to maintain samples indefinitely (such as to maintain an inventory of examples of known or new alleles), it is recommended to cryopreserve lymphocytes with DMSO and store in a liquid nitrogen freezer or to generate an EBV transformed lymphoblastoid cell line and store several aliquots in liquid nitrogen.

DNA isolation methods vary considerably in the starting material which they require in order to prepare high molecular weight DNA of sufficient purity to allow HLA class I and class II typing. Since the heme proteins of the red blood cell are known to inhibit the PCR process, many DNA isolation methods require red cell removal prior to DNA extraction. Also, cryopreservation materials such as DMSO may be inhibitory and should be washed out before DNA isolation. Below are procedures for 1) processing white blood cells from various volumes of whole blood and 2) washing cryopreserved cells.

A. Precautions

1. Blood should be handled with the appropriate precautions to avoid exposure to infectious agents.
2. EDTA or Citrate (ACD) anticoagulant is preferred. Heparin has been shown to inhibit some PCR methods. Blood samples older than one week may produce poor yields and/or poor quality DNA unless they have been stored frozen.

3. Yield is dependent on the white cell count of the sample. One ml whole blood has about 10 million white cells and yields ~100 µg DNA suitable for ~200 amplification reactions.
4. During the preparation of genomic DNA, care should be taken to avoid any contamination with previously amplified DNA.
   a. To prevent contamination, two separate locations should be used, one dedicated for pre-PCR manipulations e.g., DNA isolation and PCR set up, and the other location for manipulations of PCR-amplified DNA fragments. Each location should be equipped with its own set of pipettes, lab coats, and other materials. The pre-PCR manipulations should be handled in a laminar flow hood, if possible, to decrease the possibility of contamination.
   b. Disposable gloves should be worn. New, sterile disposable plastic tubes or autoclaved glassware should be used.
   c. All the reagents should be freshly prepared and/or autoclaved, if appropriate.
5. DNA extraction is the first step in DNA typing methods. Preparations of high quality DNA are critical for amplification of class I alleles since the length of the amplified fragment is in the range of 1000 base pairs.
6. Polypropylene tubes and tips should be used for isolating DNA; other plastic products may absorb DNA.
7. The need to store prepared DNA for long times should be considered in the choice of a protocol. For example, reference DNA used to monitor the specificity of primers and probes may be utilized over a long period of time. Therefore, a protocol that produces a more purified DNA preparation should be selected.

B. Preparation of Cells for DNA Isolation

DNA preparation methods listed below include protocols to prepare white blood cells from anti-coagulated whole blood or buffy coat preparations by red blood cell lysis. If a commercial DNA isolation method is selected which specifically states that it can isolate PCR amplifiable DNA from whole blood without a red cell lysis step or that it is compatible with heparinized samples, this process of red cell lysis may be omitted. This procedure should result in a slightly pinkish pellet of white blood cells. In general, it is necessary to perform 2 cycles of red cell lysis in order to remove the majority of the heme contamination. In addition, if heparin has been used as a blood anti-coagulant, this 2 cycle wash process will also remove the heparin, which is known to inhibit the PCR process.

C. Phenol/Chloroform Extraction: 11th HLA Workshop Protocol

This method produces very pure DNA, which can be stored for long periods of time. The method uses phenol, which is hazardous and is more time-consuming than other protocols. There has been one report that DNA prepared in this fashion might not bind well to all membranes.

Reagents, Supplies, and Equipment

1. **White Cell Lysis Buffer: WCLB**
   10 mM Tris- HCl (pH 7.6)
   10 mM EDTA (pH 8.0)
   50 mM NaCl
2. **Red Cell Lysis Buffer: RCLB**
   10 mM Tris-HCl (pH 7.6)
   5 mM $MgCl_2$
   10 mM NaCl
3. **PCI** (Phenol-chloroform-isoamyl alcohol): use within a month after preparation
   a. Mix 3 volume of melted phenol with 1 volume of chloroform-isoamyl alcohol (24:1).
   b. Add 0.6 volume of 1 M Tris-HCl (pH 8.0) and mix well.
   c. Spin 10 min at 1500 rpm or let stand until the phenol phase becomes transparent.
   d. Remove the upper aqueous phase.
   e. Add 0.6 volume of 0.1 M Tris-HCl (pH 8.0) and 8-hydroxy-quinoline to a final concentration of 0.1 %.
   f. Mix well.
   g. Spin 10 min at 1500 rpm or stand enough time to obtain transparency of the phenol phase.
   h. Remove the upper aqueous phase.
4. **10% SDS**
5. **Proteinase K** (Merck) 10 mg/ml, dissolved in dH_2O
   a. Store at -20°C until use.
   b. Incubation at 37° C for 30 minutes may be necessary for auto-digestion.
6. **TE buffer:** 10 mM Tris-HCl mM EDTA (pH 8.0)

Procedure

DNA Source and Cell Lysis

1. **Fresh Peripheral Blood**
   a. Collect 10 ml of blood for one individual and mix with 2 ml of 5% EDTA (pH 7.4)
   b. Spin the mix 5 min. at 1500 rpm
   c. Remove the upper phase of plasma without touching to the leucocytes
   d. Collect the buffy coat and transfer to a new 15 ml Falcon tube (No. 2097)

e. Add 10 ml of RCLB and mix well
f. Spin 10 min at 1500 rpm
g. Discard the supernatant
h. Resuspend the pellet in 3 ml of WCLB
i. Add 50 µl of 10% SDS and 50 µl of Proteinase K solution
j. Shake gently horizontally (50 rpm), overnight at 42° C to 50° C

2. **Frozen Peripheral Blood**
   a. Freezing: obtain the buffy coat and store at -80° C
   b. Thaw quickly and add 10 ml of RCLB, and then proceed as above
3. **Cell Lines and Frozen Lymphocytes**
   a. Pellet 20 to 50 x 10^6 cells in a 15 ml Falcon tube
   b. Resuspend in 3 ml of WCLB
   c. Add SDS and Proteinase K and incubate for 3 hours to overnight at 42° C to 50° C.

Phenol Chloroform Extraction

1. Add 3 ml of PCI
2. Shake 10 min in a tridimensional shaker or by gentle hand shaking (a complete emulsion must be obtained)
3. Spin 5 min at 1500 rpm
4. Transfer the upper aqueous phase into a new 15 ml Falcon tube.
5. Make a second PCI extraction
6. Extract once with chloroform-isoamyl alcohol (24:1) and recover the aqueous phase.

Isopropanol Precipitation

1. Add 60 µl of 5 M NaCl to obtain a final concentration of 0.1 M
2. Add 0.6 volume of 100% isopropanol
3. Mix gently by inversion until the precipitate is formed
4. The precipitation is achieved when the precipitate floats
5. Recover the DNA precipitate in a 5 ml sterile Falcon tube
6. Rinse 3 times with 3 ml of 70% ethanol
7. Dry 10 min. in vacuum
8. Resuspend in TE buffer (0.1 to 1 ml depending on the DNA precipitate amount).
9. To help this resuspension use a rotative agitator several hours or incubate at 42° C
10. Adjust DNA concentration to 100 µg/ml. The concentration of DNA should be checked on an agarose gel stained with ethidium bromide by comparison with a known quantity of phage lambda DNA, because the estimation of DNA concentration by OD measurement may be inaccurate due to contamination of RNA during the preparation. Alternatively, the DNA concentration may be measured in a fluorometer.

    Storage: 4 ° C for daily use; – 80 ° C for several years; -20° C is acceptable.

D. Salting-Out Procedure:

Miller et al. Nucleic Acids Research 16: 1215, 1988 as modified by L.A. Baxter-Lowe and K.W. Lee. Contributed by Carolyn Hurley.

Reagents, Supplies, and Equipment

1. **Red blood cell lysing solution**
   Sterile, distilled H_2O 1.0 L
   NH_4Cl 7.5 g
   Tris 1.0 g
   Dissolve NH4Cl and Tris in water. Adjust pH to 7.2. Keep refrigerated. Shelf life is approximately 6 months.
2. **Nuclei lysis buffer**
   10 mM Tris pH 8.2
   400 mM NaCl
   2 mM EDTA
   pH 8.2 with HCl
   For 100 ml: 10 ml 1M Tris, pH 8.0, 0.8 ml 5M NaCl, 0.4 ml 0.5 M EDTA pH 8.0, 90 ml dH_2O. The pH is adjusted with either HCl or NaOH to 8.2 .
3. **10% SDS** *CAUTION* – This reagent is extremely harmful if inhaled; do not autoclave.
4. Proteinase K, 2mg/ml in dH_2O. Stored frozen.
5. 5.3 M NaCl
6. Ethanol, 100% and 75% (v/v dH_2O), cold
7. Vortex, Centrifuge, Microfuge, Water bath (65°C), Pipettor with tips, Polypropylene tubes

Procedure

Note: For smaller or larger sample sizes, modify the reagent volumes proportionally.

1. Add 500 µl-1 ml of whole blood or buffy coat to 1 ml cold (0°C to -5°C) Red Cell Lysis Buffer. Vortex for 30 sec.
2. Centrifuge for 1 min at 10,000-12,000 x g and pour off red supernatant. Blot rim of tube on a paper towel. A white to red pellet remains in the bottom of the tube.

3. Add 2 ml Red Cell Lysis Buffer, vortex for 3-4 sec to resuspend pellet. Centrifuge for 30 sec at 10,000-12,000 x g. Drain off all fluid. The pellet remaining should be white to pink. This step should be repeated as necessary until pellet is white to pink.
4. For every 10-20 million white cells in pellet, add 200 µl Nuclear Lysis Buffer and 50 µl 10% SDS to each pellet.
5. Break up pellet with pipet tip and vortex to get powdery, tiny flakes.
6. Add an additional 150 µl Nuclear Lysis Buffer and vortex.
7. Add 100 µl Proteinase K (2mg/ml), mix but do not vortex.
8. Incubate at 65°C for 2 hr.
9. Add 175 µl 5.3 M NaCl and centrifuge at top speed for 15 min in microfuge.
10. Transfer supernatant to fresh tube.
11. Add 1 ml cold 100% ethanol to supernatant. Invert 6-10 times to precipitate DNA. It will appear as a white to translucent stringy mass. Centrifuge 10 min at top speed to pellet precipitate.
12. Pour off supernatant, being careful not to lose pellet. Wash the pellet with 1 ml cold 75% ethanol (break pellet by tapping) and centrifuge again 1-2 minutes at highest speed.
13. Pour off ethanol and air dry with the cap open to evaporate the ethanol, or briefly (1-2 min.) dry in a vacuum centrifuge.
14. Store as dry pellet (in -20°C).
15. Dissolve the pellet in 100-200 µl sterile distilled water. Put in 65°C water bath for 15 min to dissolve the DNA sample. Use gentle vortexing to resuspend. If clumps of undissolved DNA are present, return to 65°C until completely resuspended.

E. Method Using Trimethylammonium Bromide Salts: Protocol described by Gustincich et al. BioTechniques 1991, 11:298-302 as slightly modified by Olerup, with permission for the 12th IHW.

Reagents, Supplies, and Equipment

1. **12% DTAB Solution**
   12% dodecyltrimethylammonium bromide
   2.25 M NaCl
   150 mM Tris-HCl, pH 8.6
   75 mM EDTA
   Store at room temperature
2. **5% CTAB Solution**
   5% cetyltrimethylammonium bromide or hexadecyltrimethylammonium bromide (Cetrimide)
   0.4 M NaCl
   Store at room temperature
3. **Additional chemicals needed:**
   Chloroform
   1.2 M NaCl
   99.5 % ethanol
   70% ethanol

Procedure

Note: This protocol will yield purified DNA suitable for PCR amplification in less than 30 minutes.

1. Add 450 µl of 12% DTAB solution to 450 µl of EDTA or ACD-blood (fresh or frozen) in a 2 ml tube
2. Mix gently by inversion, 15 sec.
3. Incubate in a waterbath at 68° C for 5 minutes
4. Add 900 µl chloroform (room temperature, RT).
5. Mix immediately by vigorous inversion for 15 sec.
6. Centrifuge 10,000 rpm, 2 min.
7. Dissolve the DNA-CTAB pellet in 300 µl 1.2 M NaCl
8. Add 750 µl 99.5 % ethanol (RT)
9. Let the DNA precipitate.
10. Spin down the DNA (13,000 rpm, 2 min.)
11. Wash once with 70% ethanol
12. Spin down the DNA (13,000 rpm, 2 min.)
13. Pour off the ethanol
14. Wash once with 70% ethanol
15. Remove the remains of ethanol by wiping the walls of the tube with a cotton-tipped applicator
16. Dissolve the DNA in 50-200 µl dH_2O by vortexing for 20 sec.
17. Measure the DNA concentration by optical density reading. If you are experienced in molecular biology, you may omit measuring the DNA concentration and adjust the amount of dH_2O to the size of the DNA pellet.
18. Adjust the DNA concentration to 40 ng/ml.
19. Use 2 µl of the DNA solution for each 10 µl PCR reaction.

II. Commercial Vendors

There are many vendors which offer self-contained "kits" for PCR-ready DNA extraction from fresh/frozen whole blood, buffy coat, lymphocytes, plasma, serum, body fluids, cultured cells, cell suspensions, bone marrow, dry blood spots, and tissues from various sources. Cost of kits vary, and will be a major determinant in choosing to continue performing DNA extractions with in-house solutions versus commercially QC'd preparations. In general, those kits containing solid-phase chemistry are significantly more expensive per sample than others on the market. Inclusion of enzymes such as proteinase K within a kit also increases the price. The commercial products available can be separated into two general categories according to the method of DNA separation:

A. Solid Phase
B. Solution Phase

A. Solid Phase DNA Separation

Affinity Column Separation

1. QIAamp Blood Kit: Contributed by Jennifer Ng
(QIAgen, Inc., 1-800-426-8157 [USA] or 02103-892-230 [Germany])

This system provides a rapid extraction and purification of DNA for direct use in PCR amplification. DNA is extracted from the cell nucleus by lysis of the cell and nuclear membranes, occurring by a chemical reaction. A proteinase enzyme degrades proteins bound to the DNA. The extracted DNA is separated and trapped in an affinity column by osmotic selection. The DNA is released by washing with an eluant that solubilizes the DNA. The protocol provided with the product should be utilized for DNA preparation with the following modifications.

Reagents, Supplies, and Equipment

1. Whole Blood, Frozen PBLs
2. QIAamp Kit
3. 1.5 ml microcentrifuge tubes
4. Centrifuge
5. Water bath or hot block
6. Ultra pure water
7. Mixer (vortex)
8. Pipettes
9. Bio-hood

Procedure

1. Extractions are performed in the pre-PCR hood under sterile conditions. Transfer sample into the pre-labeled 1.5 ml microfuge tube.
   For whole blood (if frozen, let thaw) : Aliquot 200 µl.
   For PBLs (if frozen, let thaw): Spin at 14,000 RPM for one minute in its original vial. Pipette off supernatant. Add 200 µl of PBS. Vortex for 30 seconds. Transfer.
2. Lightly vortex Proteinase Stock Solution or Protease Stock Solution, and add 25 µl to sample. Vortex AL Buffer thoroughly and add 200 µl to sample. Vortex sample for 15 seconds.
3. Incubate at 70°C for 10 min. At this time add a microfuge tube filled with Distilled Water to 70°C incubator in preparation for STEP 9. Prepare and label a QIAmp-spin column and two collecting tubes per each sample.
4. Add 210 µl of ethanol (96-100%) and mix again by vortexing.
5. Apply the lysate to a QIAmp-spin column. Centrifuge at 8,000 RPM for 1 minute.
6. Discard the collecting tube together with the filtrate. Place the spin column in a clean collecting tube. Wash with 500 µl of AW Buffer. Centrifuge at 8,000 RPM for 1 minute.
7. Pour off the filtrate from the collecting tube and replace the spin column in the same collecting tube. Wash again with 500 µl of AW Buffer. Centrifuge at 14,000 RPM for 3 min.
8. Discard the collecting portion of the spin column together with the filtrate. Place the spin column in the fully labeled microfuge tube. Elute the DNA with 200 µl of distilled water that was preheated to 70°C. Spin at 8,000 RPM for 1 min.
9. Yield of genomic DNA depends on the number of cells in the sample. A 200 µl sample of whole blood from a healthy donor ($5x10^6$ leukocytes/ml) will yield approximately 6 µg of DNA, while 10^7 lymphocytes or cultured cells will yield up to 50 µg of DNA.
10. **Reference:** January 1997 QIAamp Blood Kit Handbook QIAGEN GmbH, QIAGEN Inc., all rights reserved.

2. QIAamp 96 Spin Blood Kit: Contributed by Marcelo Fernandez – Vina

This protocol describes the procedure for preparing genomic DNA extracts using the QIAamp 96 Spin Blood Kit. The QIAamp 96 Spin Blood Kit may be used for whole blood, plasma, serum, bone marrow, body fluids, lymphocytes or cultured cells.

Reagents, Supplies, and Equipment

1. QIAamp 96 plate
2. 2 ml deep-well plates (2)
3. 1 ml round well block (QIAgen)
4. Plate cap
5. Strip caps
6. Titer-Top™ plate sealing films
7. **Buffer AL**
   a. Prepare Buffer AL by decanting all of Reagent AL1 into the bottle labeled Buffer AL (Reagent AL2).
   b. Mix thoroughly by shaking.
   c. Mark the cap indicating that the reagent is complete. Buffer AL is stable for 1 year when stored in the dark at room temperature.
8. **Buffer AW**
   a. Add the appropriate amount of ethanol (96-100%) to Buffer AW concentrate before using the reagent for the first time. The amount is described on the bottle, but is currently 640 ml for the 24 plate kit size.
   b. Mark the cap indicating that ethanol has been added.
   c. Buffer AW is stable for 3 months when stored at room temperature.
9. **QIAgen Protease**
   a. Add the appropriate amount of distilled water to the bottle containing lyophilized protease. This is labeled on the bottle, but is currently 7.0 ml of distilled water to each bottle containing 140 mg of lyophilized protease.
   b. Once prepared, the protease is stable for 3 months when stored at 4°C.
10. Distilled water
11. Ethanol (96-100%)
12. Reagent reservoirs for multichannel pipet
13. Multichannel pipette with tips
14. Single pipette with tips (Rainin pipetman P200, P1000)
15. Sigma 6-10 centrifuge

Procedure

Notes before starting:

- Equilibrate the samples to room temperature.
- Equilibrate distilled water to 70°C for elution.
- Preheat a 70°C incubator for later use.
- Ensure that adequate Buffer AL, Buffer AW, distilled water, and QIAgen protease have been prepared as directed.
- If a precipitate has formed in Buffer AL, dissolve it by incubating at 70°C.

1. Mark a 2 ml deep well plate with the batch number and pipet 25 µl QIAgen protease stock solution into each of the wells.
2. Mix each sample and add it to the deep-well plate without moistening the rim of the wells. Use either 200 µl whole blood, plasma, serum, or body fluids per well, or 10^7 lymphocytes or cultured cells in 200 µl PBS per well. Samples must be added in the same order as written. Do not add sample to the negative control wells, but add all reagents throughout the procedure. NOTE: Care should be taken throughout to avoid wetting rim of wells.
3. Add 200 µl of Buffer AL to each of the wells. Seal the wells using the plate sealing film.
4. Vigorously mix the plate on a plate shaker at least 20 seconds.
5. Incubate at 70°C for 15-20 minutes in an incubator. Record the temperature, time in, and time out for this first incubation.
6. Immediately remove the plate sealing film before any condensation accumulates.
7. Add 210 µl of ethanol to each well.
8. Reseal the wells using a clean plate sealing film. Vigorously shake the plate for 20 seconds.
9. Place a QIAamp 96 plate on top of a 2 ml deep well plate. Be aware of orientation. Mark the QIAamp 96 plate with the batch number for later identification.
10. Apply all of the mixture (635 µl per well) to the QIAamp 96 plate. Remove the plate sealing film. Be careful to maintain the correct sample order during this transfer. As the samples are ordered but not labeled, an error may be undetectable.
11. Cover the QIAamp 96 plate with the plate cap provided. Mark the plate cap for later identification. Centrifuge at 6000 rpm for 3 min.
    NOTE: If only one batch of samples is being extracted, it will be necessary to prepare a balance plate for all centrifugation steps.
12. Remove the plate cap and keep inverted for use in later steps. Add 500 µl of Buffer AW to each well.
13. Cover the QIAamp 96 plate with the same plate cap in the same orientation as used previously. Centrifuge at 6000 rpm for 1 min.
14. Remove the plate cap and keep it inverted. Add 500 µl of Buffer AW to each well.
15. Again, cover the QIAamp 96 plate with the same plate cap. Centrifuge at 6000 rpm for 3 minutes.
16. Remove and invert the plate cap. Then, incubate QIAamp 96 plate at 70°C for 15 min in an incubator to dry the membrane. Keep the QIAmp 96 plate upside down and away from the bottom and sides of the incubator where the heating coils may melt the plastic. Record the temperature, time in, and time out for this second incubation.

17. Place QIAamp 96 plate on top of a clean 1 ml round well block in the correct orientation. To elute the DNA, add 200 µl of distilled water preheated to 70°C to each well and cover the QIAamp 96 plate with the same plate cap. After incubating for 1 min at room temperature, centrifuge at 6000 rpm for 3 min. Use strip caps to seal the wells of the plate for storage.

3. Generation Capture Column (Gentra, 1-800-866-3039)

a. Single sample kit

b. 96-well format kit (on the market in 1999)

i. Sold for use with fresh/frozen whole blood, bone marrow, buffy coat, plasma, body fluids, cultured cells, cell suspensions
ii. No pre-lysing step, sample is directly loaded onto column
iii. No ethanol precipitation step, DNA is eluted off column freed from cellular impurities
iv. EDTA is best anticoagulant
v. 200 µl sample volumes
vi. 15 min. prep time

NOTE: Gentra recommends using much less DNA per reaction (<10% of PCR reaction volume) and eluting using their buffer instead of ddH_2O to avoid acid hydrolysis of DNA during storage.

Magnetic Bead Separation

4. Dynabeads DNA Direct (Dynal AS, 1-800-638-9416)

Single tube

1. Isolation can be performed on:
   a. fresh (do not keep at 4°C > 1 week), frozen, and dried whole blood (5-10 µl)
   b. bone marrow (1-5 µl)
   c. cultured cells (10^5 is maximum for the system)
2. Method is based on absorption of DNA onto magnetic beads during cell lysis with subsequent washing and resuspension directly on the beads without the need for centrifugation or use of organic solvents
3. All anti-coagulants are compatible, however, heparin gives slightly poorer extractions
4. The recommended sample volumes yield enough DNA for approximately 10 PCR reactions (using 10% of DNA product per reaction)
5. Sample processing time of approximately 10 min.

Auto96 Format

1. Approximately 1 min. processing time per sample if whole tray is used and fully automated (Biomek compatible)
2. 20 µl blood yields enough DNA for 20 PCR reactions (5 µl per reaction)

5. FTA Gene Guard System (Fitzco, 1-800-367-8760)

The FTA™ Gene Guard System consists of a treated paper card on which liquid blood can be collected and stored in a dried state indefinitely at room temperature. When the blood is spotted on the FTA card, the cells are lysed and DNA is immobilized within the matrix of the paper. A small paper punch of the blood stain is prepared and processed by washing with FTA Purification Reagent and TE buffer. This removes heme and other cell debris while simultaneously purifying the bound DNA sufficiently so that the paper punch taken from the original blood spot containing the bound DNA can be used directly in a PCR reaction without further isolation or quantification of the DNA.

Reagents, Supplies and Equipment

1. FTA purification reagent (Gibco LTI, Rockville, MD)
2. 2 ml Spin-EASE tubes (Gibco LTI, Rockville, MD)
3. 1X TE Buffer (10mM Tris, 1mM EDTA, pH 8) (Gibco LTI, Rockville, MD)
4. Sterile dH_2O
5. 2 mm paper punch (Harris Micro-Punch™, Gibco LTI, Rockville, MD)

Procedure for PCR Analysis of liquid blood

1. Make a 2 mm punch from blood stain applied on the FTA-coated paper and place into the basket of a Spin-Ease tube. (See Notes 1 and 2)
2. Apply 200 µl FTA purification reagent to the blood stained paper (the paper punch will swell and most of the solution will flow through into the extraction tube). (See Note 3)
3. Cap tube and vortex 3-5 seconds.
4. Centrifuge tubes in a microcentrifuge at full speed (e.g., 12,000 x g) for 30 seconds. Discard wash solution.
5. Repeat steps 2,3 and 4 for a total of two washes with FTA purification reagent.
6. Add 200 µl of TE buffer and vortex 3-5 seconds.
7. Centrifuge samples at full speed for 30 seconds and discard filtrate.
8. Repeat TE wash step a total of two times.
9. Place the punch directly into the PCR reaction tube used for amplification. For the 2 mm punch use a PCR reaction volume of 50 µl. (See Note 4)

Notes:
1. An alternative method is to add the punch directly to the PCR tube and perform the washing in the PCR tube. Be sure that all residual TE buffer is removed.
2. Since the DNA is bound tightly to the FTA matrix after the blood is completely dried, it is not necessary to rinse the punch used to cut out a sample between uses. Make sure no residual paper is carried from one punching to the next.
3. Do not substitute other reagents for the FTA Purification Reagent in the washing steps.
4. Do not elute the DNA from the punch, as this will result in loss of DNA.

B. Solution Phase Only

1. **Pel-Freez** (1-800-558-4511)
   a. Fresh/frozen whole blood, buffy coat (heparin **NOT** recommended)
   b. Modified salting-out; pre-lyse with detergent, ethanol precipitation
   c. 6-14 µg DNA from 500 µl of whole blood, or 30-40 µg DNA from 500 µl of buffy coat isolated from 2 ml of whole blood
   d. Indicated processing time is 45-60 min., although the 65°C digestion step is routinely increased from the recommended 10 min. to a maximum of 1 hr., giving a final processing time of approximately 2 hrs.
2. **Puregene DNA Isolation Kits** (Gentra, 1-800-866-3039)
   a. Very similar to Pel-Freez
   b. Kits available:
      i. Whole blood, bone marrow
         1. Compatible with all anticoagulants, however EDTA is recommended
         2. Pre-lysing step, ethanol precipitation
         3. 200 µl sample volumes
         4. 25 minute processing time
         5. Short term storage (< 6 mo.) at 4°C, archival storage at -20°C
      ii. Clotted blood
         1. 50 µl clot homogenized with lysis buffer
         2. overnight Proteinase K incubation
         3. follows whole blood extraction procedure
         4. average DNA yield from 17-39 µg DNA per ml of blood
      iii. Buccal cells
         1. isolate cells with buccal brush
         2. hydrate DNA with 20 µl buffer, use 2.5 µl DNA for PCR
         3. brushes can be stored at room temperature for up to 4 weeks before extraction
3. **InstaGene** (Bio-Rad, 1-800-2-BIO-RAD)
   a. Pre-lysing step
      i. With ethanol precipitation
         1. Whole blood (genomic and smaller fraction DNA), dry blood on filters (PCR *in situ*)
      ii. Without ethanol precipitation
         1. Whole blood
   b. Compatible with all anticoagulants
   c. 300 µl whole blood sample volumes
   d. 30 min. prep time
4. **Bio-Fast** (Bio-Synthesis, 1-800-227-0627)
   a. Whole blood
   b. Pre-lysing step, isopropanol precipitation
   c. 500 ml whole blood sample volumes (heparin and EDTA are acceptable anticoagulants)
   d. 30 min. processing time

III. Other Commercial Kits and DNA Preparation Methods

The above are not the only methods that can be used for DNA preparation. Other methods and commercial kits may also provide DNA which can be amplified by PCR. Laboratories should examine the yield of DNA, the ability to routinely amplify the DNA to a quantity sufficient for typing, and the long term degradation of the DNA preparation in determining the optimal DNA preparation method. Different sample types may require different protocols. Because the class I amplicon is longer than the class II amplicon, the DNA preparation method is more critical to the success of the PCR.

IV. Interpretation

DNA extracted by any of these procedures is ready to use in the PCR. Usually it is not necessary to check the quality or amount of DNA, but this can be done spectrophotometrically or by gel electrophoresis. Briefly:

A. SPECTROPHOTOMETRY: The DNA is diluted in water (dilution ratio 1:50 to 1:200) and then its absorbance is measured in a spectrophotometer at the UV wavelengths of 260 nm and 280 nm. DNA concentration is calculated from the formula:

Absorbance 260 nm x dilution factor x 50 = µg/ml DNA

The ratio:

Absorbance 260 nm/Absorbance 280 nm = 1.8 to 2.0 indicates that the DNA is free of cellular contaminants. Values above this range indicate the presence of protein and membrane fractions.

B. YIELDS: About 0.5 x 10^6 white cells have a DNA content of 3µg. Bases on this, one ml of whole blood should yield between 30 and 70 µg DNA. In practice, about half this amount has been reported for the Salting-out procedure.

C. QUALITY: The Sucrose-Triton and Chelex protocol yield DNA that is still contaminated with cellular constituents such as protein and membrane fractions. When such DNA is assayed spectrophotometrically, it will show a low ratio of A260 to A280(less than 1.75) indicating that protein is present. The Salting-out method yields a DNA preparation that is free of contaminating proteins with a spectrophotometric ratio between 1.8 and 2.0.

V. DNA Amplification

A. THERMAL CYCLING

1. Monitoring Thermal Cycler

A thermocouple should be used to measure well temperatures on a routine basis to insure proper functioning of the cycler.

VI. DETECTION OF AMPLIFIED DNA

Note: The following are two very popular methods for gel electrophoresis for detection of amplicons. With commercial kits, a specific gel electrophoresis protocol is often required. Make sure that the gel electrophoresis protocol you use will be compatible with the kit.

A. Agarose Gel Electrophoresis: Contributed by Jennifer Ng

The success of a PCR amplification can be judged by running a small aliquot of the amplified material on a 1.2% agarose gel and detecting the amplicons by gel electrophoresis and fluorescence staining with ethidium bromide. DNA is negatively charged due to its phosphate backbone, therefore DNA will migrate in an electric field toward the positive pole. The distance of migration is determined by the size of the DNA fragment; small fragments move faster than large fragments. If the DNA has amplified correctly and specifically, a single ethidium bromide stained band should appear at the approximate location for that number of base pairs flanked by the chosen primer sets (depends on the type of amplification). A commercially prepared set of DNA markers is used to determine the fragment size. At least 5 µg of genomic DNA is required, and can be detected by staining with the fluorescent dye ethidium bromide. Genomic DNA is often too viscous to enter the gel, and if that is the case it can be sheared by three cycles of freeze-thaw or by passing three times through a 21-gauge syringe needle.

Reagents, Supplies, and Equipment

1. **Gel Loading Buffer:** Make with sterile solutions but do not autoclave
   For 6X buffer:
   0.24% (w/v) bromophenol blue
   0.24% (w/v) xylene cyanol
   30% (v/v) glycerol
   To make 100 ml of buffer:
   0.24 g bromophenol blue (BioRad Cat# 161-0404)
   0.24 g xylene cyanol (BioRad Cat# 161-0423)
   30 ml glycerol
   ddH_2O to a final volume of 100 ml
   Filter the buffer through a 0.2-micron filter. Aliquot 1.5 ml/tube and store at 4°C.
2. **1X TBE Gel Buffer**
   Add 10 ml 10X TBE stock solution
   Bring final volume to 100 ml with ddH_2O
3. **Ethidium Bromide** (*CAUTION*-Carcinogenic) – Make with sterile solutions but do not autoclave
   (i) Make EtBr from Tablet (Bio-Rad #161-0430); 5 mg/ml in sterile ddH_2O. Store at 4°C in a brown and/or aluminum foil-wrapped glass bottle.
   (ii) EtBr from concentrated 10 mg/ml (Bio-Rad #161-0433); Keep it in a cool, dark place.
4. **Agarose** (Boehringer Mannheim #9012-36-6)
5. **1 Kb DNA ladder** (e.g., GibcoBRL #15615-016)
6. **Equipment:** Hot/stir plate, gel apparatus, power supply, short wave UV box, camera, Micro-titer plates (V-bottom, Sardstedt #82.1583.001)

Procedure

1. Preparation of agarose gel.
   1.0% (w/v) agarose (2.0 g) in 200 ml 1X TBE buffer. Place on stirrer to mix. Heat to boiling in a microwave. (1 min per 100 ml) [**Note:** Do not over boil, as too much water will evaporate.] Remove bottle with protective gloves and add 8 µl of 5 mg/ml ethidium bromide. Place on stirrer to mix, and pour into apparatus.
2. Place 4 combs (28 wells) into gel. Allow to solidify for 30-60 min. Avoid bubbles in gel and make sure it is level during pouring and when cooling.
3. Fill gel tank with 1X TBE buffer; gel is submerged in buffer at least 5 mm.
4. Add 1 µl of 6X gel loading buffer to each well of an empty 96 well V-bottom tissue culture tray. Add 5 µl of amplified DNA and mix up and down three times.
5. Store remaining amplified DNA at 4°C.
6. Prepare molecular weight markers by mixing 10 µl of 1 Kb DNA ladder with 156.7 µl ddH_2O. Vortex thoroughly and add 5 µl of the diluted ladder to 1 µl of the 6X loading buffer. Load 6 µl marker in the appropriate wells.
7. Close top of gel apparatus. Connect electrodes, minus (-, black) near samples, plus (+, red) at the far end.
8. Run at 240 volts (CV) for 45 min. exactly. Dye should separate towards the positive end into two colors. Do not run dye off gel. Do not exceed 260 volts. DNA can not separate well with higher voltage or shorter running time. DNA ladder could be degraded at higher voltage.
9. Wearing gloves and UV safety glasses, remove gel from buffer and take a photo on the short wave UV transilluminator.
10. If a robust band at appropriate size (e.g., 1000 bp HLA-B generic amplification) is not visible (either weaker than the majority or non-existent), repeat the amplification starting with DNA already prepared using different concentrations. (i.e.1 µl, 2 µl, 3 µl of DNA) Don't confuse the faster migrating primers, which also stain with ethidium bromide for the amplified DNA.

B. Preparation and Electrophoresis of 96 Well Agarose Gel: Contributed by Derek Middleton
Note: for 96 Well PCR Plates

Reagents and Supplies

1. **10X TBE:** For 2 liters: Mix 216 g Tris, 110 g Orthoboric Acid and 80 ml 0.5M EDTA to 1400 ml dH_2O. Adjust volume to 2 liters with dH_2O. Sterilize by autoclaving.
2. **Cresol Red** (10 mg/ml):Vol 20 mls: Measure 200 mg (0.200 g) into weighing boat. Dissolve in some of dH_2O taken from measured 20 ml dH_2O in a sterile universal. Resuspend in remaining volume. Filter sterilize and dispense into 1 ml aliquots in 1.5 ml eppendorfs. Freeze at -20°C.
3. **1M Tris** pH 7.6: Vol 2 liters: Add 242.28 g Tris base in parts to 1400 ml dH_2O. Adjust the pH to 7.6 by adding 100 ml concentrated HCl. *CAUTION:* Wear a mask and goggles and, where possible, do this job in a fume hood. Allow the solution to cool to room temperature before making the final adjustments to the pH. Sterilize by autoclaving. Notes: If the 1M solution has a yellow color, discard it and obtain better quality Tris. More than 100 ml concentrated HCl may be required.
4. **0.5M EDTA** pH 8.0: Vol 1 liter: Add 186.1 g of EDTA $Na_2 2H_2O$ in parts to 800 ml dH_2O. Adjust the pH to 8.0 using 4M NaOH. Make up to 1 liter with dH_2O. (Alternatively approximately 20 g NaOH pellets can be substituted for 4M NaOH). Sterilize by autoclaving.
5. **10% SDS** – Sodium Dodecyl Sulphate. *CAUTION* – This reagent is extremely harmful if inhaled. Wear a mask when working with SDS powder. Also wear gloves. Wash skin throughly if in contact with SDS. Wipe down work area after use. Preferably add SDS to dH_2O in fume hood. Vol 1 liter: Add 100g of SDS in parts to approximately 800 ml dH_2O. Apply a little heat (up to 68°C) if necessary to assist dissolution. Allow to cool to room temperature and then adjust the volume to 1 liter. Do NOT autoclave.
6. **Sucrose** (Supplier BDH).
7. **Gel – Loading Buffer** (GLB): Vol 50 ml

Stock	*Final Conc*	*Amount Required*
1M TRIS (pH 7.6)	20 mM	1 ml
0.5M EDTA	20 mM	2 ml
10% SDS	0.2%	1 ml
Cresol Red	0.04%	0.02 g
Sucrose	16%	8 g

   Add 10 ml dH_2O to a 50 ml Falcon tube. Add 8 g sucrose (slowly) and mix by inversion until dissolved. Then add Tris, EDTA, SDS and cresol red. Make up to 50 ml with dH_2O, mix and store at room temperature. Do NOT autoclave.
8. **Tris-EDTA** (TE) **Buffer** (10 mM Tris/1 mM EDTA pH 7.6): Vol 1 liter: Combine the following reagents: 10 ml Tris (1M) pH 7.6, 2 ml EDTA (0.5M). Make up to 1 liter with dH_2O. Sterilize by autoclaving. Once sterlized, aliquot into pre-labeled bijoux.

Procedure for Preparation of Gel

1. Prepare 300 ml of 1X TBE by combining 30 ml of 10X TBE buffer with 270 ml of ddH_2O in a 500 ml graduated cylinder.
2. Transfer the 300 ml of 1X TBE solution to a 500 ml conical flask.
3. Add 4.5 g of agarose and swirl the flask to mix. Heat in a microwave for 2 minutes on high. Remove and swirl to mix. Return to microwave and heat until boiling.
4. When the agarose solution begins to boil, remove the flask from the microwave and place a thermometer into flask. It is important to stir gel while cooling to prevent "lumps" forming.
5. While gel is cooling, prepare 96-well-gel template by placing template (such as the Jordan Scientific Co. template sealer) – taking care to ensure the template is not over tightened otherwise it will crack when gel is added.
6. Place sealed template on top of a leveling table and set "spirit level" into middle of template. Adjust the feet of the leveling table until the bubble in the "spirit level" is centered. Remove "spirit level".
7. Once gel has cooled to 65°C, remove and clean thermometer with tissues. Add 15 µl of ethidium bromide (10 mg/ml) and mix gently using thermometer. (Eject tip into sharps box after use and clean thermometer with tissues to ensure no ethidium bromide remains on it).
8. Pour the molten agarose solution into the level gel template (from step 6). Immediately push any air bubbles to edges of the template using a pipette tip – again dispose of into a sharps box.
9. Insert four 24 slot combs into the gel, with equal spacing between combs – Ensure white edge of comb is facing you – to allow longest space for DNA to migrate on gel. Allow gel to set for approximately 1 hour at room temperature.

Electrophoresis

1. Prepare 1000 ml of 1X TBE by combining 100 ml of 10X TBE buffer with 900 ml of ddH_2O in a 1 liter graduated cylinder. Cover mouth of cylinder with parafilm and mix well by inversion.
2. Add 1000 ml of 1X TBE to 96 Well gel electrophoresis tank. Carefully remove combs from gel. Remove gel from the gel sealer. Place gel in tank containing 1 x TBE buffer. **Note:** Ensure gel is covered by buffer to a depth of 2.3mm. Add more buffer if necessary.
3. Add 8 µl of GLB into each well of 96 well PCR plate containing 4 µl of product using a 1-10 µl multichannel pipettor (Anachem Octapipette). Change filter tips between each addition of TE/GLB mix. **Note:** PCR product is dispensed using dot blotting machine.
4. Once GLB has been added to all the wells, spin plate in centrifuge for 1 min at 1000 rpm.
5. Prepare 40 µl of 1 x 174/Hae III size marker by combining 20 µl of GLB with 12 µl (1.2 µg) of marker and 8 µl of TE Buffer in a 1.5 ml microcentrifuge tube.
6. Load 10 µl of size marker into first well of each of the four rows.
7. Using the 1-10 µl multichannel pipettor, carefully load 10 µl of sample into each well of the gel. Care must be taken to ensure the pipettor is orientated properly when adding the samples to the gel.
8. Place the lid of the electrophoresis system on to the electrophoresis tank, connect the electrodes to the power pack (Gibco BRL Model 400L) and electrophorese the samples at 250 V, 250 mAmp for 20 mins. The samples should be electrophoresed from the negative anode towards the positive cathode.
9. Once electrophoresis is complete, remove the gel from the tank and photograph using UV light.

C. DNA Quantification Using PicoGreen® Dye: Contributed by Marcelo Fernandez-Vina

PicoGreen® binds to double stranded DNA, but not single stranded DNA or RNA. This protocol may replace routine electrophoresis to assess PCR amplification success. A small aliquot of each amplification is mixed with the PicoGreen® dye. After a short incubation, sample fluorescence is measured using a fluorometer. Fluorescence is directly proportional to the quantity of double stranded DNA present.

Reagents, Supplies, and Equipment

PicoGreen® dye (Molecular Probes)
TE buffer (10mM Tris, 1mM EDTA, pH 7.5)
Microfluor plates
Pipet tips
Pipettors
50 ml screwtap tubes
Pipets
Fluorscan II fluorometer
Aluminum foil

Procedure

1. **Fluorscan II Operation**
   a. Turn on the Fluorscan II fluorometer at least 1 hour before starting the procedure.
   b. Set both the excitation filter and the emission filter to setting 2. The excitation filter should be 485 nm and the emission filter should be 538 nm.
2. **PicoGreen® Preparation**
   a. Bring the PicoGreen® reagent to room temperature.

b. Prepare a working solution of the PicoGreen® dye by diluting it 1:200 in TE buffer. For example, to prepare enough for two 96 well plates of PCR amplifications, add 100 µl PicoGreen® reagent to 19.9 ml of TE buffer in a 50 ml screwtop cap.
c. Wrap the tube containing the working solution of PicoGreen® dye in aluminum foil to protect it from light.

3. **DNA Detection**
   a. Add 90 µl of TE buffer to each well of a microfluor plate.
   b. Add 10 µl of PCR amplified product to the corresponding wells of the microfluor plate and mix.
   c. Add 100 µl of working solution of the PicoGreen® reagent to each well and mix by tapping plate gently.
   d. Cover the plate with aluminum foil and incubate for 5 minutes at room temperature.
   e. After incubation, measure the sample fluorescence using the Fluorscan II.
   f. Attach the printed assay results to the PCR sheet.
4. **Cleaning of Microfluor Plates**
   a. After the test is completed, rinse the plates with deionized water, making sure that the wells are completely flushed.
   b. Soak the washed microfluor plates in 10% bleach overnight.
   c. Rinse the bleached microfluor plates 3-4 times with deionized water, making sure to fill each well completely.
   d. Blot the microfluor plates on some paper towels and allow to dry.
5. **Results**
   The results should be validated locally as the OD value will vary depending on the size of the PCR product and the efficiency of the primers. Usually, the fluorescent reading of each amplicon must be at least 3 times the reading of the PCR negative control. The presence of primer-dimers is suggested if the fluorescent reading of the negative control is as high as the amplicons.
6. **Handling and Storage**
   a. PicoGreen® is stored frozen at -20°C. It must be brought to room temperature before use. It must be protected from light. When making the working solution, protect from light by wrapping the tube in foil.
   b. Currently, there are no data on the toxicity or mutagenicity of the PicoGreen® reagent. However, since it binds to double stranded DNA, it should be treated as a possible mutagen. In addition, dimethyl sulfoxide (DMSO) is known to facilitate the uptake of organic molecules into tissue. Therefore, caution should be exercised when using this reagent. Double gloves are recommended when handling the DMSO stock solution.

VII. Troubleshooting

The major concern with DNA extraction is the failure to obtain DNA that can be used for PCR amplifications. This could be due to a problem with:

1. the **QUANTITY** of DNA
2. the **QUALITY** of DNA

Using the Sucrose/Triton procedure, we have found that amplification failures can often be rectified by using a smaller volume (i.e., 2 µl instead of 5 µl) of the DNA extract in the PCR. It appears that too much DNA inhibits PCR, possibly by diluting out the primer. Sometimes the problem is too little DNA, and that is easily solved by using more extract in the PCR. Less than 100 ng of DNA in a 100 µl reaction has sometimes been amplified.

Amplification failures can also occur because DNA is contaminated with cellular protein and membrane fractions. In this case, the DNA has a low A_{260} / A_{280} ratio when measured by a spectrophotometer. DNA with a ratio as low as 0.7 has been successfully amplified, so it does not appear that PCR requires extremely clean DNA, at least for the oligotyping protocols. “Dirty” DNA can always be cleaned up by using the Salting-out extraction procedure outlined above, starting from step 4.

The real problem, however, seems to be when there is too much (or too little) DNA and the DNA is too “dirty”. Such DNA does not amplify even on dilution (or on increasing the amount) and must be cleaned up for successful amplification.

Acknowledgements

1. The Sucrose/Triton procedure was modified from a protocol from Dr. Barbara Schmeckpeper, National Red Cross Histocompatibility Laboratory, Baltimore, MD.
2. The Chelex procedure is courtesy of Dr. Gayle Rosner.
3. The Salting-out procedure is modified from a protocol from Dr. David Bing of the Center for Blood Research in Boston.
4. The use of PCR-DK buffer to extract DNA from buccal mucosa and hair follicles is courtesy of Ms. Rita Glumm of the Blood Center of S.E. Wisconsin.

A number of colleagues have contributed extensively to refining our in-house protocols. We also wish to recognize the members of the 13th IHW for extensive contributions to the present manuscript:

Carolyn Katovich Hurley
Marcelo Fernandez-Vina
Xiaojiang Gao

Derek Middleton
Jennifer Ng
Harriet Noreen
Ee Chee Ren
Barbara Schmeckpepper
Anjane Smith
Ting Tang
Katsushi Tokunaga

References

1. Sambrook j, Fritsch EF, Maniatis T: Molecular cloning: a laboratory manual. New York: Cold Spring Harbor Laboratory Press, 1989.
2. Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA Struhl K: Current protocols in molecular biology. New York, John Wiley and Sons, 1992.
3. Beutler E, Gelbart T, Kuhl W: Interference of heparin with the Polymerase Chain Reaction. BioTechniques 9:166, 1990
4. Kawasaki ES: Sample preparation from blood, cells and other fluids. In: PCR protocols: A guide to methods and application. Innis MA, Gelfand DH, Sninsky JJ, White TH, eds. New York: Academic Press, p146, 1990.
5. Higuchi R. Simple and rapid preparation of samples for PCR. In: PCR Technology: principles and applications for DNA amplification. HA Erlich, ed. New York: Stockton Press, p 31, 1989.
6. Singer-Sam J, Tanguay RL, Riggs, AD. Use of Chelex to improve the PCR signal from a small number of cells. In: Amplifications: A forum for PCR users. Perkin-Elmer Cetus, 1989.
7. Walsh PS, Metzger DA, Higuchi R: Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. BioTechniques 10:506, 1991
8. Miller SA, Dykes DD, Polesky HF: A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Research 16:1215, 1998.

Primers and Probes: Design and Labeling for Detection of Nucleic Acids

Deborah Crowe

Principle

All nucleic acid-based assays rely on the detection of differences in the base sequence between different genes or alleles. This usually involves only a small segment of the total DNA present in the cell. The first step in DNA typing is to find the "needle in the haystack," i.e., to determine the presence or absence of the target sequence in the midst of multiple other sequences. This is routinely accomplished by DNA:DNA or DNA:RNA hybridization. DNA hybridization can be extremely specific and sensitive, depending upon hybridization conditions. The hybridization reaction is often supplemented with either specific fragmentation using restriction endonucleases or by using the polymerase chain reaction (PCR) to amplify a region of interest.

The second step in DNA typing is to determine if hybridization did take place. Detection of hybridization can be done in several ways, but usually involves labeling primers or probes. The most frequent reporter groups in use are ^{32}P, fluorescent dyes, or signal generating enzymes like alkaline phosphatase (AP) or horseradish peroxidase (HRP). The signal that they generate can be detected with methods such as autoradiography, scintillation counting, fluorescence microscopy, colorimetry, or changes in chemiluminescent substrates. For years, the highest sensitivity was obtained from ^{32}P labeled probes. However, several non-isotopic systems have been developed which are as sensitive and are now routinely used for HLA typing purposes. For this reason, this chapter will deal mainly with non-isotopic methods of labeling.

The most widely used non-isotopic method utilizes AP or HRP with chemiluminescent substrates. This method produces flashes of light in a dark background and can be easily detected by direct exposure to high speed film or sensitive luminometers. HRP is less expensive, easier to use, and seems to have less stearic hindrance since it is much smaller than AP. HRP has more initial activity, but less long term activity than AP, since it is auto-inhibited by its substrates. The greatest advantage of using AP is that it affords better control over signal intensity by varying the time exposed to either substrate or X-ray film.

Background

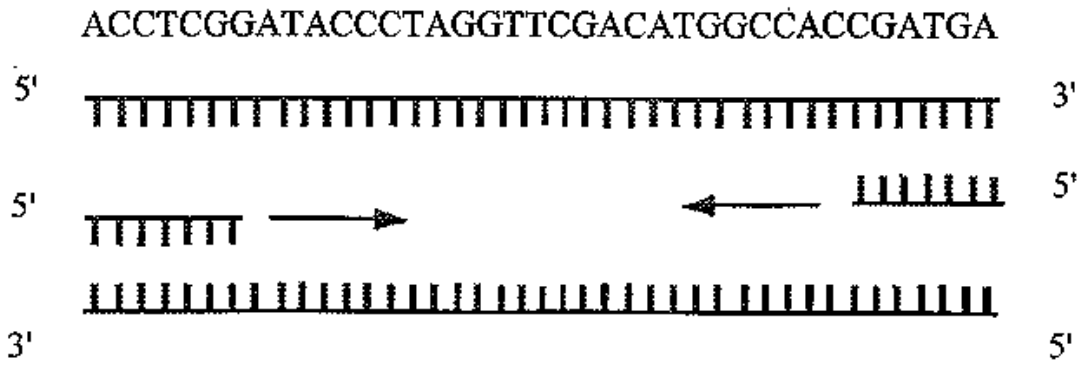
DNA consists of two complementary polymorphic chains of nucleotides twisted in a right-handed double helix. Each nucleotide has three parts: deoxyribose sugar, phosphate, and a purine or pyrimidine base. The purines found in DNA are adenine and guanine and the pyrimidines are cytosine and thymine (uracil in RNA). The sequence of bases encodes the genetic information. The strands are anti-parallel, which means that they run in opposite 5′ to 3′ directions. The anti-parallel orientation of the two helices favors the optimal association for hydrogen bond formation between specific complementary bases. Base pairing between the opposite strands occurs between purines and pyrimidines, with adenine binding to thymine, and guanine binding to cytosine. Pairing between purines and pyrimidines (and not between two purines and two pyrimidines) is crucial for maintaining a constant distance between the two chains and providing optimal stability to the double helix.

There are two hydrogen bonds between A and T and three hydrogen bonds between C and G. Thus, it requires more energy to break bonds between the C and G. DNA sequences which are G-C rich will have a greater melting temperature. The bases are linked to the C-1 of the deoxyribose sugar at the N-9 of purines and at the N-1 of pyrimidines. The base pairing is the basis for DNA:DNA and DNA:RNA hybridization. Primers and probes used in DNA typing are designed to have complementary bases to known sequences of interest.

By convention, the DNA strand that codes for the protein is written in the 5′ to 3′ direction. The 5′ end of the gene sequence includes the control regions and the amino terminus of the protein. The 3′ end includes the stop codon and the carboxyl terminus of the protein. Nucleotides are attached to each other through a 3′-5′ phosphodiester bonds, with the phosphate group at the 5′ end of the next nucleotide attaching to the 3′ carbon of the deoxyribose of the previous nucleotide in the chain. Therefore, when designing primers, the 3′ end is the most important because the new nucleotide is added to the 3′ end of the primer. Sequence-specific priming (SSP) takes advantage of this property by placing the polymorphic difference at the 3′ end of the primer. Only DNA containing the complementary sequence will be primed by the specific primer.

I. Primer Design

Primers are short oligonucleotides that are necessary for amplification of DNA in the PCR reaction. The primers anneal to specific regions of denatured DNA. The DNA polymerase then catalyzes the attachment of subsequent nucleotides to the free 3′ OH of the growing strand, using the complementary strand as a template. Primers are designed to flank the sequence of interest so that after approximately 30 PCR cycles, there is approximately a million-fold amplification of the target DNA. Each primer set consists of 2 primers – one which flanks the sequence of interest on the 5′ end, and the other which flanks the sequence on the 3′ end. In this way, both strands of DNA will be replicated during the PCR cycling.



The 5′ primer has the same sequence as the DNA sequence and will prime the complementary strand. DNA replication is always in the 5′→3′ direction. Since the 3′ primer must replicate in the opposite direction, it will be complementary and opposite from the written gene sequence. In the example above, the 5′ primer will read the same as the written sequence (5′ ACCTCGGA) while the 3′ primer will read (5′ TCATCGG), which is complementary and in the opposite direction from the 3′ end of the sequence. Since DNA sequences are always written 5′→3′, it is important to remember when ordering primers to write both the 5′ and the 3′ primers in the 5′→3′ direction.

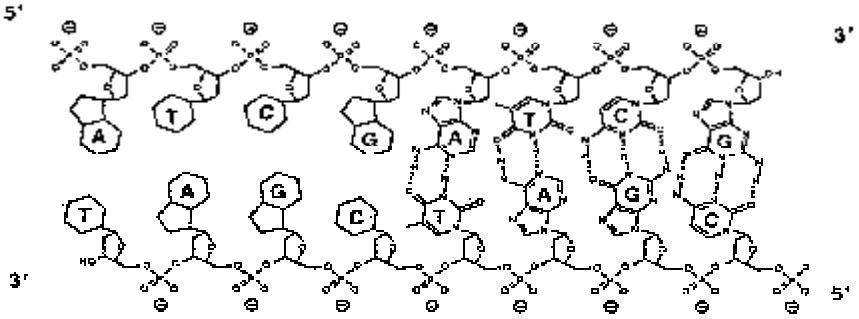


Figure 2: DNA Complementarity is due to constraints of Distance and Hydrogen Bonding

Use of Primers with Probe Hybridization Methods

In probe hybridization methods for DNA typing, it is necessary to first amplify the gene sequence of interest. For example, most of the polymorphism for Class II molecules lies in exon 2 of the gene sequence. Primers are designed to flank the entire sequence for exon 2 and this region is amplified by PCR. Then the PCR product is tested with a panel of probes that will detect the polymorphic differences in the sequence that differentiates the different alleles. For this type of testing, only one primer set is usually needed. The primers are complementary to consensus regions so that all samples are amplified with the same primer set.

II. Probe Design

A. Probes are usually designed so that the sequence for the polymorphism being examined is placed in the center of the oligonucleotide probe. One can visualize this as creating a “bubble” when the probe tries to hybridize with a sequence that is not complementary to the polymorphic site. When exposed to stringent conditions and washes, the probe will come off much easier if there is a “bubble”.

B. The length of the probe is usually around 20 bases. This can be modified somewhat in order to get the probe to anneal under the same conditions as other probes in the panel. The optimal annealing temperature is dependent upon length and GC content.

C. The sequence of the probe should be examined to ensure that it will not form “hairpins” or have “end-to-end” hybridization.

D. If a probe is having problems with specificity, it can be re-designed to move the polymorphic site either up or downstream. It can also be made longer or shorter to affect the Tm and optimal annealing conditions.
E. GC content: should be 40-60%, if possible

III. Use of Primers with Sequence-Specific Primer (SSP) Methods

Another approach to DNA typing for HLA is to use the primers themselves as probes. The primers can be designed to be complementary to specific polymorphic regions. If the sample being tested has the specific polymorphism, it will be amplified. If it does not have the polymorphism, it will not be amplified. (See SSP chapter). This method requires many primer sets since each primer set will amplify a different allele or group of alleles. Much more PCR testing is required for this method, but it is usually faster because no additional testing with a panel of probes is needed. Since the Taq polymerase catalyzes the addition of new nucleotides to the 3′ end of the primer, the bases at the 3′ end of the primer are most important for conferring specificity. ***Therefore, when designing primers for SSP, the primers should include the polymorphic bases at the 3′ end.***

There are several rules and recommendations to keep in mind when designing primers. A good primer will retain both specificity and efficiency of amplification. Below is a summary of the key points that one should consider when designing primers.

1. **Primer Sequence**
   a. 3′ end is critical for specificity
   b. Avoid runs of 3 or more G or C residues at 3′ end – decreases specificity
   c. Avoid T at 3′ end – more prone to mispriming
   d. Check primer pairs for complementarity which may result in primer-dimer formation or hairpin secondary structure; Primer-dimers can cause a decrease in sensitivity or even failure of amplification of the specific product
   e. GC content: should be 40-60%, if possible
2. **What to do if these problems with primer sequence cannot be avoided**
   a. Experiment with different PCR buffers
   b. Optimize $MgCl_2$ concentration
   c. If critical base is a "T", try putting it second to 3′ end instead of at 3′ end
   d. Insert mismatch upstream from 3′ end if getting cross-hybridization
   e. Add DMSO (10-15% final volume) to PCR buffer to increase fidelity of priming
3. **Primer Length**
   a. 18-30 bases is optimal
      18 bases should be unique among 7×10^{10} base pairs and should only hybridize at one position in most eukaryotic genomes.
   b. Different primers to be assayed together in single panel should have similar Tm values.
4. **T_m – melting temperature**
   a. $T_m = 2°C \times (A+T) + 4°C \times (C+G)$
   b. T_m should be 55 – 65°C

IV. Preparation of Primer and Probe Solutions

A. Calculation of Primer or Probe Concentration

The Primers are usually received lyophilized and are reconstituted by adding 100-200 µl ddH_2O. Vortex. Incubate at 37°C for 20 minutes. Vortex.
This is the **Stock Oligo.**

Some manufacturers will give you the µg quantity. The concentration of your stock would be the given µg/volume when reconstituted. (Ex. 350 µg lyophilized = 350 µg/0.1 ml when reconstituted = 3500 µg/ml.)
If µg is not known, you can determine it using a spectrophotometer:

Make a 1:200 dilution by adding 2.5 µl of the stock oligo to 500 µl ddH_2O. Read the OD at 260 nm.

OD260 _____ x 200 x 33 = µg/ml oligo of stock oligo

B. Molecular Weight and Calculation of picomoles/µg

MW is sometimes given when an oligo is purchased. If not, you can determine as follows:

Base	#Bases in oligo		Grams/base		Total Grams
A	_____	x	313.22	=	_____
C	_____	x	289.19	=	_____
G	_____	x	329.20	=	_____
T	_____	x	304.19	=	_____
				Subtotal	_____
					-61.96 grams

Total MW = _____gm/mole

pmoles/µg = 10^6/MW = _____

C. Calculation of picomoles/µl in stock oligo

_____µg/ml oligo x _____ pmoles/µg x 10^{-3} =_____ pmol/µl oligo
(from A) (from B)

D. Preparation of Working Concentration of Oligo

Desired concentration of Working Primer Oligo = 25 pmol/µl
Desired concentration of Working Primer Oligo = 50 pmol/µl
Need to calculate how much ddH_2O needs to be added to 10 µl stock oligo to make the desired concentration.

Example: If Stock oligo was 303.5 pmol/µl, then 10 µl would contain 3035 pmol.
If you wish to make a 25 pmol/µl oligo:

$$\frac{25 \text{ pmol}}{1 \text{ µl}} = \frac{3035 \text{ pmol}}{x \text{ µl}}$$

x = total µl working oligo; x – 10 µl = µl H_2O to add to 10 µl stock

(Note: pmole/µl = (M)

V. Target DNA

Purified high molecular weight DNA is required for most DNA typing procedures.
Three ratios can be used to assess purity of DNA:

1. 260/280: should be 1.65 – 1.8
   Low ratio indicates contamination with protein (aromatic amino acids)
   High ratio indicates possible contamination with RNA
2. 260/270: should be at least 1.2 to indicate an acceptably low level of phenol in the purified DNA.
3. 260/230: should be at least 2.0 to indicate acceptably low level of contamination from peptides.

VI. Nucleic Acid Labeling

Except for SSP methods, most DNA-based assays utilize a specific probe which is a short oligonucleotide that is complementary to the sequence of interest. The major challenge to DNA testing is to design probes and testing conditions that favor a strong signal for detection while maintaining very specific hybridization.

The SSOPH method uses labeled probes. PCR product is blotted onto membranes, and then hybridized with different probes. If the sample has the sequence which is complementary to the probe, hybridization takes place. The label allows one to determine whether hybridization occurred. Most newer methods and commercial kits use a reverse SSOPH method. In the reverse method, the primer is labeled instead of the probe. Unlabeled probe is blotted onto membranes or onto wells of a microtiter plate. The sample is added and if hybridization takes place, it can be detected by adding reagents which react with the label on the PCR product. Both primers and probes are short oligonucleotides. Therefore, labeling for either primer or probe would be similar. The labeling molecule itself may interfere with functional properties of the oligonucleotides. Labeled probes may not hybridize as well or may require that conditions be changed. Labeled primers may interfere with the polymerase chain reaction.

Labeling probes usually requires that an additional group be added to or substituted for portions of the DNA molecule. These substitutions may affect the specificity of the hybridization. In early days of DNA testing, radioactive labels such as ^{32}P or ^{35}S were used to label probes. These labeling molecules are very small and do not have much affect on hybridization properties. Today, because of cost and safety concerns, most laboratories are having to turn to non-radioactive labels. While safer to use and more cost effective, these labels tend to be bulkier and may be more likely to affect behavior during hybridization. Therefore, care must be taken to determine the best label for the specific use.

Probes may be labeled at the 5′ end, the 3′ end, or internally. Short probes are usually labeled at one of the ends, since end labeling is less likely to interfere with specific hybridization. Longer probes may be labeled internally by substituting or modification of internal nucleotides. Internal labeling will often provide better specific activity.

A. Direct and Indirect Labeling Systems for Nucleic Acids

In order to determine if hybridization has occurred, the probe must be labeled. Labeling systems can be classified as direct or indirect. With direct labeling, the reporter group is directly attached to the probe. This has the advantage of having fewer steps in the detection process. However, each probe must be modified before use. With indirect labeling, the probe modification is simple and the same for all probes. It can often be done during synthesis of the probe by the DNA synthesizer. The reporter groups most commonly used are ^{32}P, fluorescent dyes, or signal generating enzymes like alkaline phosphatase (AP) or horseradish peroxidase (HRP).

Examples of direct labels include the addition of ^{32}P at the 5′ end by T4 polynucleotide kinase, fluorescein substituted for internal nucleotides during sequencing, or a horseradish peroxidase covalently coupled via a N-ethyl maleimide derivative to an oligonucleotide produced with a 5′ end sulfhydryl group during automated synthesis (see Figure 3).

The most popular indirect labels are biotin and digoxigenin. Biotin is incorporated in the 5′ end during synthesis. It is then detected after hybridization by reaction with streptavidin covalently linked to HRP. Another popular method for indirect labeling involves the addition of a tail to the oligonucleotide probes using a terminal deoxynucleotidyl trans-

ferase. During formation of poly-A tail, a small percentage is substituted with digoxigenin-modified uracils. The reporter group is detected after hybridization of the probe by using an anti-digoxigenin Fab fragment covalently linked to alkaline phosphatase (see Figure 4). Both biotin and digoxigenin-based systems offer excellent alternatives to radioactive labeling. Biotin is vitamin H and since it is a natural constituent of cells, it may have a high background in some samples. Digoxigenin is a plant alkaloid that is not found in animal cells and has been shown to be as sensitive as biotin.

B. Incorporation of Modified Nucleotide Triphosphates (NTP or dNTP)

Enzymes are needed to catalyze the incorporation of modified NTP. One can use enzymes that are involved in DNA repair or synthesis. The NTPs can be modified with ^{32}P, digoxigenin or flourocein. The modified nucleotides or analogs cannot inhibit the enzyme activity and the final probe must retain both sensitive and specific hybridization properties.

For long probes, the DNA is "copied" or "repaired" from a template using methods such as nick translation, random priming and PCR. The label is incorporated homogeneously throughout the new DNA molecule.

Nick translation uses Dnase I to nick one strand of dsDNA, generating free 3′ OH ends within the unlabeled DNA. *E. coli* DNA polymerase I is then added to remove nucleotides from the 3′ side of a nick and simultaneously add new nucleotides to the 3′OH terminus of the nick. The end result is that the old nucleotides are removed and new, labeled nucleotides are added. This is possible since this enzyme has a 5′→3′ exonuclease activity in addition to the polymerase activity. This method can be used to uniformly label both strands of a dsDNA. It works well with both isotopic and non-isotopic labels.

Random priming is based on the polymerase activity of the Klenow fragment. The Klenow fragment is the C-terminal end (70%) of the entire E. coli polymerase which retains the polymerase activity and a 3′→5′ exonuclease activity, but lacks the 5→3′ exonuclease activity. The DNA is denatured and allowed to reanneal in the presence of random-sequence hexamers, which serve as primers for the DNA polymerase activity. The hexamers contain all four bases in every position. These are available from commercial companies (Promega Corp., Pharmacia, Boehringer Mannheim). The DNA product is synthesized exclusively by primer extension. Both isotopic and non-isotopic labels can be used and the resulting product is uniformly labeled.

Two methods are used most commonly to label short probes. dNTPs can be incorporated into the 3′ end by using terminal deoxynucleotidyl transferase. Modified nucleotides, such as fluorescein-dUTP, digoxigenin-11-dUTP, or biotin-14-dATP can be added on a 3′ tail in this way. T4 polynucleotide kinase is used to add a phosphate group onto the 5′ end of double or single stranded DNA. Only an isotopic label (e.g., adenosine 5′ [^{32}P] triphosphate) can be incorporated with this procedure.

C. Automated Synthesis of DNA Oligonucleotides Using Phosphoramidite Chemistry

DNA oligonucleotides are synthesized in the 3′→5′ direction, since the 5′OH is more reactive than the 3′OH. The bases are protected during synthesis in order to prevent extraneous reactions. The first nucleotide is pre-attached to a solid phase support and each nucleotide is added in a step-wise fashion using the same set of chemical reactions:

Deblock: The 5′OH group is made available by removing the dimethly trityl (DMT) protecting group with acid.

Activate and Couple: Automated synthesizers add phosphoramidite nucleotide derivatives to the growing, solid phase bound, nucleic acid chain. The phosphoramidite is rapidly activated and couples with the free 5′OH.

CAP: The unreacted hydroxyl groups are modified or "capped" to prevent them from reacting in the next cycle.

Oxidize: Trivalent phosphite is made into a phosphate by reacting with iodine. The phosphate is still protected with a cyanoethyl group.

After synthesis is completed, the oligonucleotide is removed from the solid phase with a base. All protecting groups are removed with concentrated NH_4OH. The excess reagents and salts are also removed. The final DMT group is removed either before or after cleavage from the solid phase. For some applications, a purification step may be required. Unless a phosphate is specifically added, the final product will have hydroxyl groups on both the 5′ and 3′ ends.

D. Modification of Oligonucleotides During Synthesis

Special phosphoramidites are used to place modifications at either end or within the nucleotide. Modifications are usually made to the 5′ end since internal labels are more likely to affect hybridization and 3′ end labels may interfere with the function of primers. One approach is to use biotinylated or fluorescent dye phosphoramidites which are placed directly into the growing oligo nucleotide (at either end or internally). Modifications or analogs which have the same length as the nucleotide being replaced usually will have minimal effects on hybridization.

A second approach is to use reactive groups such as primary amine or sulfhydryl groups. These are introduced into the nucleotide during synthesis. After synthesis is completed, a second reagent is used to incorporate a label into the probes. For example, at pH 8-9, primary amine groups are more reactive with N-hydroxysuccinimide esters (NHS) than exocyclic amines of A, C, and G. Several NHS-biotin, NHS-digoxigenin, and NHS-fluorescent dye derivatives can specifically react with the primary amines. Thus, NHS derivatives can be readily attached to oligonucleotides modified with the primary amine group. Similarly, at pH 6-7, sulfhydryl groups react rapidly and specifically with N-ethyl maleimide (NEM) derivatives. NEM derivatives of HRP and AP readily react with sulfhydryl modified oligonucleotides. Thus, probes which have been synthesized with primary amines or sulfhydryls can be covalently coupled to enzymes, biotin or digoxigenin in a single post-synthetic reaction.

E. Protocols

1. Digoxigenin Labeling Of Probes For SSOP – 3′ Tailing Method

Principle

This procedure uses terminal deoxynucleotidyl transferase to add a mixture of unlabeled nucleotides and digoxigenin-11-dUTP, producing a tail containing multiple digoxigenin residues. The digoxigenin serves as the antigen for an anti-digoxigenin conjugate. Upon addition of a substrate, one can detect the presence of the labeled oligonucleotide probe. The probes can detect at least 1 pg of control DNA in a dot blot assay. Tailed probes are suitable for procedures requiring optimal sensitivity.

Sample

Oligonucleotides are purchased or synthesized. They are received in desiccated form and reconstituted. A 1:200 dilution is made by adding 2.5 µl of the probe stock solution to 497.5 µl TE Buffer. The dilution is read on a UV Spectrophotometer at 260 nm wavelength against a TE buffer blank. The OD reading is recorded and the concentration in picomoles/µl of the reconstituted stock oligo is calculated. A working probe solution of 50 picomoles/µl is made and stored frozen at -20ºC until use. The remaining reconstituted stock probe is also stored at -20ºC for future use.

Reagents

DNA Tailing Kit (Boehringer Mannheim Biochemicals, Cat # 1028 707
or Genius 6, Oligonucleotide Tailing Kit (Boehringer Mannheim Cat. No. 1417 231)
or Terminal Transferase – Boehringer Mannheim Cat. No. 220 582
Supplied with:
5X tailing buffer
$CoCl_2$, 25 mM

Complete instructions come with these kits. The kits contain most of the reagents needed. Some catalog numbers for individual reagents are listed below:

Digoxigenin-11-dUTP – Boehringer Mannheim Cat. No. 1093 088
25 nmoles in 25 µl (1 mM). Use 2.5 µl per test.
dATP, 100 mM -Boehringer Mannheim Cat. No. 1051440
Make 0.05 mM (50 µM) stock from 100 mM solution by diluting the 100mM solution 1:2000 using ddH_2O
Kit may contain a 2.5 mM solution of dATP. Make a 50 µM solution by adding 4 µl of dATP to 196 µl of water.

Procedure

Note: The instructions may differ slightly, depending upon the kit used.

1. Remove $CoCl_2$, 5X buffer, and dig-11-dUTP from freezer and thaw on ice.
2. Label 0.5 ml microfuge tubes with the names of the probes to be labeled. Remove one aliquot (50 pmol/µl) of each probe to be labeled from the freezer and thaw. Make a 1:10 dilution of the probe by putting 1µl into 10 µl of deionized water. Final concentration is now 5 pmol/µl.
3. Label 1.5 ml microfuge tubes on top of cap with the probe number. Also label another 1.5 ml tube for the master mix.
4. Prepare Master Mix by combining the amount listed below x the number of probes to be labeled. (Make a little extra to account for loss in pipetting)

5X tailing buffer	5.0 µl
25 mM $CoCl_2$	5.0 µl
ddwater	1.5 µl
50 µM dATP	2.5 µl
dig-11-dUTP	3.0 µl
Terminal transferase	1.0 µl

Centrifuge for 20 seconds at full speed.

5. Aliquot 18 µl of the master mix to each 1.5 µl tube.
6. Add 7 µl of the probe dilution from #2. Centrifuge for 20 seconds at full speed.
7. Incubate tubes for 30 minutes at 37°C.
8. Add 675 µl of hybridization buffer to each probe.
9. Vortex well and store in 100 µl aliquots frozen at -20°C until needed. Avoid repeated freeze/thaw cycles.
10. The final concentration of the label is 50 pmol/ml. Each 100 µl aliquot will make 5 ml of hybridization buffer at 1 pmol/ml.
11. Before use, labeled oligos should be tested for specificity using a panel of reference DNA samples. The labeled probe should be stable when frozen for at least one year.

2. Digoxigenin Labeling of Probes for SSOP- 3′ End Labeling

1. This procedure incorporates a single digoxigenin-11-ddUTP onto the 3′ end of the oligonucleotide. The Genius 5 Oligonucleotide 3′ End-Labeling Kit (Boehringer Mannheim Biochemicals, Cat # 1362 372) supplies most of the reagents and complete instructions.

2. The reactions are set up as above for the 3′ Tailing method except digoxigenin-11-ddUTP is substituted for both the dATP and the digoxigenin-11-dUTP.
3. Perform the labeling steps as above. Once the first ddUTP is incorporated, there is no longer a 3′ end OH on the oligonucleotide and the reaction terminates.
4. Probes labeled with this method retain their high degree of specificity and can still be treated under the same optimal hybridization and washing conditions.

Quality Control for 3′ Tailing and 3′ End Labeling of Probes

DNA probes may degrade over time and lose specificity. It is important to monitor the specificity of the probes over time. Probes should be tested on a reference cell panel containing multiple examples of the alleles to be detected (or not detected).

1. After labeling, the probe is tested against a panel of known reference cells and the reactions are documented.
2. If unclear/weak/incorrect results are obtained during use of the probe, the problem is noted and corrective action is documented. If the problem cannot be resolved, the probe is re-labeled and retested.

Procedure Notes for 3′ Tailing and 3′ End Labeling of Probes

1. The number of nucleotides incorporated into the 3′ tail varies with both the concentration and type of dNTP. Longer tails are very sensitive, but can give non-specific or "fuzzy" results. For this reason, the procedure described here used less dATP and more dig-11-dUTP than suggested by the manufacturer. This results in a product that has a shorter tail but better sensitivity and work well for detection of allelic differences in PCR amplified genomic DNA bound to membranes. The ratio of dATP to digoxigenin-11-dUTP may be optimized in your lab to give the desired results.
2. The kinetics of probe hybridization should not be affected by tail length. The possibility that a poly-dA tail might anneal to a poly-dT rich region can be minimized by keeping the tail less than 15 bases or by pre-hybridizing with excess unlabeled poly-dA.
3. When examined on a polyacrylamide gel electrophoresis, tailed oligonucleotides should show a smear of heterogeneous higher molecular weight components when compared to the starting oligonucleotides.
4. 3′ end labeling with dideoxy-11-digoxigenin is 10 times less sensitive than 3′ tailing. However, it may be preferred with probes that are giving non-specific reactions with the tail labeling method.
5. Similar procedures can be used for incorporation of biotin-dNTPs into oligonucleotides.

3. Nick Translation Labeling of dsDNA

Both the Nick translation and the Random priming method incorporates labeled dNTPs into DNA using small modifications of the classic ^{32}P labeling method.

Reagents

1. 10x Dig DNA Labeling Mixture
   - 1mM dATP
   - 1mM dCTP
   - 1mM dGTP
   - 0.35mM Dig-11-dUTP
   - 0.65mM dTTP
   - pH 6.5 (+20°C)
2. 10x Reaction Buffer
   - 0.5M Tris-HCl, pH 7.5
   - 0.1mM $MgCl_2$
   - 10mM DTE
3. Dnase I / DNA Pol I
   - 0.08 mU/µl Dnase I
   - 0.1 U/µl DNA Pol I
   - 50mM Tris-HCl, pH 7.5
   - 10mM $MgCl_2$
   - 1mM DTE
   - 50% (v/v) glycerol
4. Ethanol (100%, 70%)

Procedure

1. On ice, set up the reaction as follows:

dsDNA template	1-2 µl (2 µg)
10x Dig DNA labeling mixture	4 µl
10x Reaction Buffer	4 µl
Dnase I / DNA PolI	4 µl
Add deionized water to final volume of 40 µl	

2. Incubate 15°C for 40 minutes.
3. Stop by adding 4 µl of 0.4M EDTA solution and heating at 65°C for 10-15 minutes.

4. Precipitate and dissolve the DNA as follows:
   a. Add 0.15 volume of 3M sodium acetate and 2.5 volume chilled 100% ethanol.
   b. Allow to precipitate at -70°C for 1 hour.
   c. Centrifuge 12,000 x g for 5 minutes. Discard supernatant.
   d. Quickly rinse the pellet with 70% ethanol. Dry under a vacuum for 15 minutes.
   e. Dissolve the labeled DNA in 50-100 µl deionized water or TE buffer.
5. Store at -20°C. The labeled probes must be denatured before use by boiling for 12 minutes and quickly chilling on ice for 4 minutes.

4. Random Priming Labeling of dsDNA

Reagents

1. 10x Random Hexanucleotide Mixture
   0.5M Tris-HCl, pH 7.2
   0.1M $MgCl_2$
   1mM DTE
   2 mg/ml BSA
   62.5 A260 units/ml Random Hexanucleotides
2. 10x Labeling dNTP Mixture
   1mM dATP
   1mM dCTP
   1mM dGTP
   0.35mM Dig-11-dUTP
   0.65mM dTTP
   pH 6.5
3. Klenow DNA Pol I, 2 units/µl, labeling grade
4. 3M Sodium Acetate Buffer, pH 5.2
5. Ethanol (100%, 70%)
6. TE Buffer – 10mM Tris-HCl, pH 8.0; 1mM EDTA

Procedure

1. Denature dsDNA by boiling for 12 minutes and quickly chilling on ice for 4 minutes. Briefly spin down and place on ice.
2. Mix in a microfuge tube on ice:

Denatured template	10 µl (25 ng-3 µg)
10x Random Hexanucleotide Mixture	4 µl
10x dNTPs Mixture	4 µl
Add ddH_2O to total volume of 38µl	

3. Add 2 µl of Klenow DNA Pol I (2 units/µl)
4. Incubate at 37°C for 3-12 hours.
5. Add 4 µl 0.5M EDTA to stop reaction.
6. Precipitate and dissolve DNA as described in Nick Translation method above (Step 4)

Procedure Notes for Nick Translation and Random Priming

1. If labeling with digoxigenin, substitute digoxigenin dUTP for one-third of the dTTP.
2. If labeling with biotin, substitute biotin-derivatized dATP, dCTP, or dUTP for one half of the corresponding dNTP. Partial substitution in these ratios usually gives optimal incorporation, which is in the range of 20-40 substitutions per 1000 nucleotides, i.e., one hapten for every 25-50 nucleotides of newly synthesized DNA. Higher levels of substitution may decrease overall performance of the labeled probe.
3. A spacer is needed between the digoxigenin or biotin and the dNTP. The spacer should be at least 6 carbons long. Most commercial derivatives have a spacer of at least 10 carbons.
4. The labeled probe can be used as is, or it can be cleaned up by separating it from unincorporated dNTPs using ethanol precipitation, reverse phase or gel filtration chromatography. This may improve sensitivity and/or specificity.
5. Labeled probes can be stored dry or frozen at -20°C for at least one year. Avoid repeated freeze/thaw cycles.
6. It is possible to reuse the hybridization buffer containing the labeled probes up to five times. The solution should be stored at -20°C between uses. Heat at 60°C for five minutes prior to use.
7. Commercial kits are available for non-isotopic labeling by nick translation or random priming. The kits contain most of the reagents required and should come with detailed instructions.

Limitations

1. The biotin and/or digoxigenin incorporated into short oligonucleotides can change the properties of that oligonucleotide, causing it to behave differently during ethanol precipitation and reverse phase schemes.
2. Internal incorporation of non-isotopic label should only be used when labeling long probes or DNA. Because of the bulky nature of the label, internal labeling may interfere with proper hybridization of the probe.

5. Non-Isotopic Labeling of Oligonucleotides Using Chemical Modification

A. Direct Incorporation of Oligonucleotdes during Automated Synthesis

1. As discussed above, automated synthesizers using phosphoramidite chemistry proceed from the 3′ end to the 5′ end and the end product has hydroxyl groups on both ends. Labels can be added during routine synthesis at any internal sequence or at the 5′ end. If the starting solid phase column has biotin or fluorescein already attached, it will be incorporated into the 3′ end.
2. For short oligo probes that will be used to detect small sequence differences, 3′ or 5′ labeling is preferred because it will have less effect on hybridization.
3. For PCR primers, the label should be placed at the 5′ end because the 3′ end is critical for successful amplification.
4. The addition of spacers between the label and the hybridization region of the oligonucleotide decreases the possibility of stearic affects. To increase strength of signal, one can incorporate multiple biotins in branched structures extending off the 5′ end of the probe.
5. Digoxigenin cannot be directly incorporated using phosphorimidite chemistry because the molecules cannot be adequately protected during synthesis.
6. The final product is stable dry at 4°C or frozen in aliquots at -20°C. Avoid repeated freeze/thaw cycles.

Procedure Notes

1. Always use fresh phosphoramidite reagents as older reagents may give lower step yields and less full length oligonucleotide.
2. It may be necessary to increase the reagent concentration or the reaction time for the coupling steps when using modified phosphoramidites, because these reagents are often less efficient than standard phosphoramidites.
3. If excellent step yields are obtained, clean-up of the product may only require desalting to remove the concentrated NH_4OH and blocking groups released after deprotection. The dimethyltrityl protecting group is hydrophobic and interacts strongly with C-18 or reversed phase solid supports. Therefore, oligos are often synthesized with trityl “on” so that the reversed phase solid supports can be used to pull out only the full length sequences.
4. If biotin or other label is placed only on the 5′ end of the oligonucleotide, only the full length probes will be able to generate a signal and false hybridization of shorter products will have minimal effect.

B. Incorporation of Reactive Groups (Amino or Thiol) into Oligonucleotides during Synthesis

1. Reactive groups themselves are not labels. However, once incorporated into the sequence, the oligo can be easily labeled. The amino group is very reactive with N-hydroxysuccinimide (NHS) and the sulfhydryl groups is very reactive with horseradish peroxidase.
2. Reactive groups (amino or sulfhydryl) can be added in two ways. One can modify the phosphorimidites with amino or sulfhydryl groups and the reactive group can then be inserted internally or at the 5′ end during synthesis. Alternatively, amino or thiol modified columns are available for 3′ end incorporation.
3. Amino-modified reagents are protected with either trifluoroacetyl (TFA) or monomethytrityl (MMT) groups. TFA is removed during standard base cleavage and deprotection, but MMT is retained and can be used for reverse phase purification. The MMT is like DMT, but the protecting groups are cleaved with acid.
4. Sulhydryl-modifed reagents come protected with a trityl group or as a disulfide with terminal dimethoxytrityl (DMT). The trityl-sulfur bond requires silver nitrate for deprotection, whereas the disulfide forms free sulfhydryl with dithiothreitol (DTT).
5. It may be necessary to increase either the reagent concentration or the reaction time for the coupling steps.
6. **Amino Modification:**
   a. Unless 3′ end modification or removal or failed sequences is required, the 5′-amino-modifier C6-TFA (Glen Research #10-1916-02) is recommended.
   b. Perform the synthesis in trityl-off mode.
   c. After completion, perform the standard cleavage/deprotection.
   d. Remove all NH_4OH and trace salts.
   e. Take up in sterile distilled water at a concentration of about 500nM (100 OD units) per ml.
   f. Store at 4°C or frozen in aliquots at -20°C.
7. **Sulfhydryl Modification:**
   a. Unless 3′ end modification or removal or failed sequences is required, the Thiol-Modifier C6-S-S (Glen Research #10-10936-02) is recommended.
   b. Perform the synthesis in trityl-off mode.
   c. After completion, perform the standard cleavage step.
   d. To the routine deprotection step, add 50mM DTT and incubate with concentrated NH_4OH as usual.
   e. Remove all NH_4OH and trace salts.
   f. Take up in sterile distilled water at a concentration of about 500nM (100 OD units) per ml.
   g. Store at 4°C or frozen in aliquots at -20°C.

   NOTE: Be aware that the DTT must be thoroughly removed before subsequent reaction steps. This can be done by desalting the modified oligonucleotide on G-25 sephadex immediately before use, or, if necessary, the DTT can be extracted from solution with ethyl acetate.

8. **5′ End Modification:**
   a. Only full length products will contain the reactive group and allow coupling of biotin, digoxigenin, fluorescent dye, or reporter enzyme to the oligonucleotide.
   b. Purification of 5′ end modified oligos may not be necessary.

C. Labeling of Amino-modified Oliogonucleotides with N-hydroxysuccinimide (NHS)

1. Dissolve 100 nM (about 20 OD units) of desalted, dry 5′ amino-modified oligonucleotide (with at least a 6 carbon spacer) in 0.7 ml sterile distilled water.
2. Add 100 µl of 1M $NaHCO_3$, pH 9.0.
3. Just before use, add 880 µl of anhydrous dimethylformamide (DMF: Aldrich #22,705-6 or Pierce #20672 G) to a 10 mg vial of biotinamidocaproate N-hydroxysuccinimide ester (NHS-biotin: Sigma # B 2643 or Pierce # 21336 G). Mix well.
4. Add 200 µl of NHS-biotin ester to the amino modified oligonucleotide in carbonate buffer, pH 9; Cover and mix;
5. Incubate at least 60 minutes at 30°C or overnight at room temperature. This is a 50-fold molar excess of NHS-biotin reagent.
6. Desalt on G-25 Sephadex to remove excess reagents; Store dry or frozen in aliquots at -20°C. Repeat freeze/thaw cycles.
7. The resulting probe can be produced in large quantities (100 nmol per reaction), are specific, and have a sensitivity comparable to 3′ end-labeled probes (10 pg)
8. One advantage of 5′-end labeled oligonucleotides is that the 3′ end is free to act as a primer for DNA synthesis reactions. This is useful in reverse SSOP procedures.

Procedure Notes

1. Smaller amounts of oligonucleotide can be labeled by scaling back all volumes. The NHS ester should remain in 20-100 fold excess.
2. NHS esters specifically react with the primary amines and, at pH 8.5-9.0, they do not significantly react with the exocyclic amines of single-stranded DNA, even at 100-fold molar excess.
3. NHS esters hydrolyze in water at a rate that is pH dependent. Since DMF is hygroscopic, it is safest to make this solution just before use. The half times for NHS esters in water and at room temperature are about 5 hours at pH 7.0, 1 hour at pH 8.0, and 10 minutes at pH 8.6. The reaction rate with amines also increases with pH so that the optimum pH for the overall reaction is around pH 9.0. Protein is often modified at pH 7.0-8.5, whereas pHs of 8.5 and 9.0 have been used for the reaction of NHS esters with amino-modified DNA.
4. Similar procedures can be used to add digoxigenin or various fluorescent dyes to amino-modified oligonucleotides.

D. Labeling of Sulfhydryl-modified Oligonucleotides with Horseradish Peroxidase

1. Add 1 ml of 100 mM phosphate buffer, pH 7.2 to a 10 mg vial of horseradish peroxidase (HRP:Sigma type VI-A, # P-6782); Mix to dissolve. This is about 250 nM of HRP per ml.
2. Add 600 µl of anhydrous dimthyformamide (DMF: Aldrich # 22,705-6 or Pierce # 20672 G) to a 5 mg vial of 4-(N-maleimidomethyl)-cyclohexane-1-carboxylic acid N-hydroxysuccinimide ester (also known as SMCC: Succinimidyl 4-(N-maleimido-methyl)cyclohexane-1-carboxylate) (Sigma # M 5525 or Pierce # 22320 G); Mix well. This is about 25 µM SMCC per ml.
   Note: Prepare this reagent just before use, as DMF is hygroscopic and N-hydroxysuccinimide (NHS) esters hydrolyze rapidly in water with half-life of 10 minutes at pH 8.6 and 5 hours at pH 7.0.
3. Add 100 µl of SMCC to the HRP vial; Mix well and cover.
4. Incubate for 60 minutes at 30°C. This is a 10-fold molar excess of SMCC over HRP. HRP has only 2-3 reactive amino groups.
5. Desalt on Sephadex G-25 equilibrated in 100 mM phosphate buffer, pH 6.5 with 5 mM EDTA. Follow the brown color of HRP, which will be found in about 2.9 ml of void volume. N-ethylmaleimide (NEM) hydrolyzes slowly in water with a half-life of about one day at 20°C in 10 mM phosphate, pH 7.0.
   Note: NEM derivatized HRP can be prepared just before use, and is probably stable as aliquots frozen at -20°C. It is available lyophilized (Pierce # 31494 G). The yield should be 200-250 nM of HRP at 100nM per ml, with 1-2 NEM groups per mole of HRP.
6. Assemble the sulfhydryl-modified oligonucleotides that are to be conjugated to HRP. These should have a concentration of about 500 nM per ml (about 100 OD per ml for a 20 mer). Sulhydryl groups can be oxidized to disulfides, and so these oligonucleotides should be stored with dithiothreitol (DTT).
7. Desalt each SH oligonucleotide on Sephadex G-25 into 100 mM phosphate buffer, pH 6.5 with 5mM EDTA. Keep to a minimal volume, but be sure to remove all the DTT, as 10% contamination can significantly inhibit conjugation.
8. HRP-oligonucleotide conjugate can be separated from excess, unreacted oligo by gel filtration on Sephacryl S-100 equilibrated with TBS, pH 7.4. Both unreacted NEM-HRP and HRP-oligonucleotide are found in the first large, brown peak and should be well separated from the excess sulfhydryl-oligonucleotide. Since the sulfhydryl-oligonucleotide is added in large molar excess, there should be very little unconjugated NEM-HRP. The final product can be stored at 4°C with 0.01% thimerosal, and in 50% glycerol at -80°C for long term storage.
   Note: Be aware that HRP is inhibited by sodium azide. Avoid repeat freeze/thaw cycles.

9. If the sulfhydryl-oligonucleotide was not added in excess, there might be unreacted NEM-HRP remaining. For the most exacting work, this can be separated from oligonucleotide-HRP conjugate by ion exchange chromatography. In most cases, removal of unreacted NEM-HRP is not necessary.
10. The concentration and quality of enzyme-oligonucleotide conjugate can be estimated by determination of the OD at 260 and 280 and calculating the 260/280 ratio. Determine the ratios of pure enzyme and oligonucleotide as standards.

VII. Detection Methods

Principle

There are many variations in the way hybridization reactions can be detected. It usually involves multiple sequential incubations: blocking, hybridization of labeled probe to target DNA or labeled target DNA to probe, localization of reporter groups to the labeled probe, and signal generation.

One of the key determinants in detection methods is the choice of the solid phase support and the effective blocking on non-specific binding to these surfaces. Examples of solid phase supports commonly used are positively charged nylon membranes or polystyrene microtiter plates. Blocking agents must be optimized for each assay system. Some assays may require two blocking steps – one to minimize non-specific DNA binding during hybridization and another for non-specific protein binding. Methods to control DNA background include the addition of detergents and/or irrelevant DNA, control of salt and solvent concentrations, and high stringency washes. Methods to control protein background include addition of detergents and high concentrations of irrelevant proteins such as serum albumin or casein.

Protocols

A. Detection of Membrane-bound amplicons using Digoxigenin-labeled Probes and Alkaline Phosphatase Chemiluminescence

Principle

Lumi Phos 530 contains Lumigen PPD and an enhancer for chemiluminescent detection of alkaline phosphatase. Enzymatic dephosphorylation of PPD produces an unstable intermediate, which decomposes to emit blue light. The signal is enhanced by the presence of flourescent micelles formed by cetyltrimethyl ammonium bromide and 5-N-tetradecanoyl amino fluorescein. The fluorescein acceptor emits a bright yellow luminescence which exposes the X-ray film.

Reagents

1. Nylon membranes- Boehringer Mannheim CO # 1209 272
2. Bromphenol blue
3. 0.4N NaOH
4. Neutralization buffer:
   1500 mM NaCl
   100 mM Na Phosphate, pH 7.4
   10 mM EDTA
5. DNA Blocking Reagent:
   750 mM NaCl
   75 mM NaCitrate, pH 7.0
   1% casein
   0.2% SDS
   0.1% N-Lauryl sarcosine
6. Wash Solution 1:
   300 mM NaCl
   20 mM NaPhosphate, pH 7.4
   2 mM EDTA
   0.1% SDS
7. Wash Solution 2 (Room temperature and 59°C):
   3 M tetramethyl ammonium chloride (TMAC)
   50 mM Tris, pH 8.0,
   2 mM EDTA
   0.1% SDS
8. Wash Solution 3:
   300 mM NaCl
   100 mM Na phosphate, pH 7.4
   2 mM EDTA
9. Protein Blocking Reagent:
   2% casein
   150 mM NaCl
   100 mM Tris
   pH 7.5

10. Wash Solution 4:
    150 mM NaCl
    100 mM Tris
    pH 7.5
11. Wash Solution 5:
    100 mM NaCl
    50 mM MgCl2
    100 mM Tris
    pH 9.5.
12. **Anti-digoxigenin Conjugate:** Prepare just before use!
    Make a 1:5000 dilution of anti-digoxigenin (Fab) conjugated to alkaline phosphatase (Boehringer Mannheim Co # 1093 274) by adding 80 µl of the stock to 400 ml of Protein Blocking Reagent.
13. Lumi Phos 530 (Boehringer Mannheim Co # 1413 163)

Procedure

1. Dot 2 µl of amplicon in a 96 well microtiter format, directly onto dry nylon membranes by taking the PCR reaction product directly into the syringes of a MicroBlot-96 apparatus (Robbins Scientific). Air dry.
   A small amount of Bromphenol blue can be added to the amplicons (final concentration of 70 µg/ml). This does not interfere with the PCR amplification and gives positive confirmation that the PCR reaction mixture was put on the membrane.
2. Denature the DNA by incubating the membranes in 0.4N NaOH for 5 minutes, followed by neutralization in Neutralization buffer for 10 minutes.
3 Air dry for 60 minutes at room temperature. Bake at 110°C for 30 minutes under a vacuum.
4. Pre-hybridize (block) membranes for 30 minutes in 5 ml of DNA Blocking Reagent at 42°C in a 50 ml plastic tube.
5. Add 5 pmol aliquot of each digoxigenin-tailed probe to the appropriately labeled membrane/tube; cover and mix well; incubate at 42°C overnight with constant rotation.
6. Wash the membranes for two minutes in 200 ml of room temperature Wash Solution 1. Repeat wash once.
7. Wash the membranes for 10 minutes in 200 ml of room temperature Wash Solution 2.
8. Wash the membranes for 15 minutes in 200 ml of 59°C Wash Solution 2. Repeat wash once.
9. Wash the membranes for 10 minutes in 200 ml of room temperature Wash Solution 3. Repeat wash once.
10. Incubate the membranes in 20 ml per filter of Protein Blocking Reagent with constant agitation at room temperature for 60 minutes.
11. Prepare 400 ml of 1:5000 anti-digoxigenin conjugate. Transfer membranes and incubate with constant agitation at room temperature for 30 minutes.
12. Wash for 15 minutes in 400 ml Wash Solution 4 at room temperature. Repeat once.
13. Wash for 2 minutes in 200 ml of room temperature Wash Solution 5.
14. Dip each membrane into a shallow tray containing 10 ml of Lumi Phos 530 at 37°C solution for 5-10 seconds; remove and drip-dry for 5 seconds; place membranes between two acetate sheets and incubate at 37°C for 30 minutes.
15. Place membranes into X-ray film cassettes and expose to film for 1-10 minutes, as needed.

B. Colorimetric Detection of Membrane-Bound Alkaline Phosphatase

Reagents

1. NBT: Prepare 75 mg/ml of NBT (Sigma # N 6876) in 70% DMF (v/v) in water. Store at 4°C for up to one year
2. BCIP: Prepare 50 ml/ml of BCIP (Sigma # B 8503) in DMF. Store at 4°C for up to one year.
3. Nylon membranes- Boehringer Mannheim CO # 1209 272
4. Bromphenol blue
5. 0.4N NaOH
6. Neutralization buffer:
   1500 mM NaCl
   100 mM Na Phosphate, pH 7.4
   10 mM EDTA
7. DNA Blocking Reagent:
   750 mM NaCl
   75 mM NaCitrate, pH 7.0
   1% casein
   0.2% SDS
   0.1% N-Lauryl sarcosine
8. Wash Solution 1:
   300 mM NaCl
   20mM NaPhosphate, pH 7.4
   2 mM EDTA
   0.1% SDS

9. Wash Solution 2 (Room temperature and 59°C):
   3M tetramethyl ammonium chloride (TMAC)
   50mM Tris, pH 8.0,
   2mM EDTA
   0.1% SDS
10. Wash Solution 3:
   300 mM NaCl
   100 mM Na phosphate, pH 7.4
   2 mM EDTA
11. Protein Blocking Reagent:
   2% casein
   150 mM NaCl
   100 mM Tris
   pH 7.5
12. Wash Solution 4:
   150 mM NaCl
   100 mM Tris
   pH 7.5
13. Wash Solution 5:
   150 mM NaCl
   50 mM MgCl2
   100 mM Tris
   pH 9.5

Procedure

1. Hybridize probe and alkaline phosphatase to DNA target as usual (see steps 1-12 above).
2. Wash membranes for 10 minutes at room temperature in Wash Solution 5.
3. Prepare enough fresh NBT/BCIP substrate solution to develop membranes, by adding 45 µl stock NBT and 35 µl stock BCIP to each 10 ml of room temperature Wash Solution. Use fresh and keep away from bright light.
4. Develop membranes in the dark or out of direct light. A dark precipitate will form in a few minutes to hours, depending on the amount of alkaline phosphatase that is present.
5. Xeroxing wet membranes produces a permanent record. The membranes can be dried, but the color becomes less intense. Store membranes in the dark; the color will fade over weeks or months.

C. Direct Detection using Enzyme-Oligonucleotide Conjugates

Oligonucleotides that are directly conjugated to the enzymes alkaline phosphatase (AP) or horseradish peroxidase (HRP) can be used to detect DNA targets.

Advantages

1. Fewer steps and washes
2. Less hands on time
3. Rapid and sensitive reactions
4. Clean backgrounds and crisp reaction patterns.
5. Once the correct blocking/hybridization/stringency conditions are found, the directly conjugated probes perform as well as ^{32}P-labeled probes.

Disadvantages

1. Each probe has to be conjugated separately.
2. Time consuming and expensive;
3. Stringency problem – each probe will have to be optimized, but the enzymes are temperature sensitive. Decreasing activity has been reported at 42°C, higher at 50°C, and excessive at 58°C. Stringency can be increased by decreasing salt concentration.

VIII. References

1. ASHI Reference Manual, "Non-Isotopic labeling and Detection of Nucleic Acids" by Michael Chopek and Mark Z. Wescott. Chapter IV.B.1.
2. ASHI Reference Manual, "Methods for Labeling DNA with Radioistopes" by Barbara J. Schmeckpeper. Chapter IV.B.2
3. Boehringer Mannheim Biochemicals. *The Genius System Users Guide for Filter Hybridization*. 1992. Boehringer Mannheim Corporation, Indianapolis, IN.
4. Savage MD, Mattson G, Desai S, Neilander GW, Morgensen S, Conklin EJ: *Avidin-Biotin Chemistry: A Handbook*. Pierce Chemical Company, Rockford, IL, 1992.
5. Ausubel FM, Brent R, Kingston RF, Moore DD, Seidman JG, Smith JA, Struhl K (eds): *Current Protocols in Molecular Biology*; Green Publishing Associates and Wiley-Interscience, NY, 1987 (with continuous updates).
6. Glen Research. *User Guide to DNA Modification and Labeling*. Glen Research Co., Sterling VA, 1990.
7. Kricka LJ (ed). *Nonisotopic DNA Probe Techniques*. Academic Press Inc. San Diego, CA, 1992.

8. Kessler C (ed). Nonradioactive Labeling and Detection of Biomolecules. Springer-Verlag. Berlin, Germany, 1992.
9. Applied Biosystems, Inc. *Evaluating and Isolating Synthetic oligonucleotides: The Complete Guide.* Applied Biosystems, Inc. Foster City, CA, 1992.
10. Beckman Instruments, Inc. *DNA Synthesis Reference Guide.* Beckman Instruments, Inc., Fullerton, CA, 1992.
11. Innis MA, Gelfand PH, Sninsky JJ, White TJ (eds): *PCR Protocols: A Guide to Methods and Applications*. Academic Press, NY, 1990.
12. Kaufman Peter B., Wu William, Kim Donghen, and Cserke Leland J., "Preparation of Nucleic Acid Probes" in *Handbook of Molecular and Cellular Methods in Biology and Medicine*. CRC Press, 1995.

PCR-SSP Typing of HLA Class I and Class II Alleles

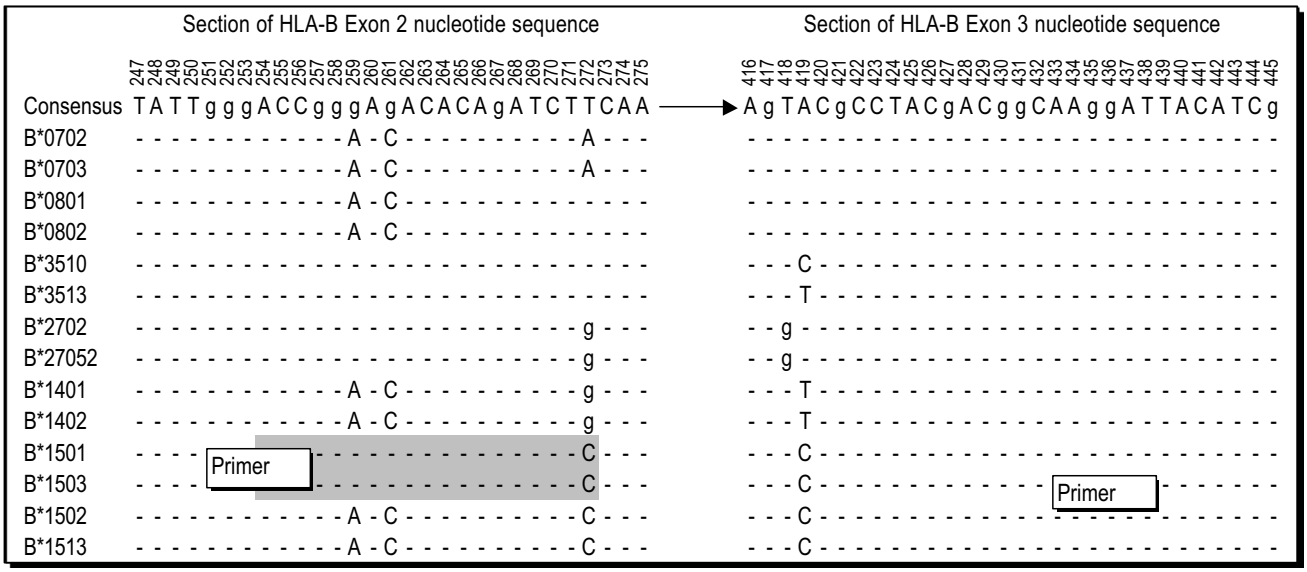
Mike Bunce and Ken Welsh

Purpose

Accurate HLA typing is required for both solid organ transplantation and bone marrow transplantation. In addition HLA typing is advantageous for many research applications and disease association studies. The use of molecular typing methods for defining HLA class I and class II alleles are now commonplace. Molecular methods offer flexibility of resolution, improved reproducibility and greater accuracy compared to traditional serological methods.[1-5] The advantages of PCR-based methods of typing have led to the widespread use of molecular typing methods even to the extent where they have totally replaced serological methods in some centers.

Most PCR-SSP systems feature multiple small volume PCR reactions where each reaction is specific for an allele, or more commonly a group of alleles which correspond to a serologically defined antigen. PCR-SSP specificity is derived from matching the terminal 3′-nucleotide of the primers with the target DNA sequence. *Taq* polymerase extends 3′-matched primers but not 3′-mismatched primers, consequently only target DNA complementary to both primers is efficiently amplified. PCR-SSP works because *Taq* polymerase lacks 3′ to 5′-exonucleolytic proofreading activity.[6, 7] Such an activity would correct the mismatched terminal base of an SSP primer in a mismatched primer-template complex and subsequently permit efficient priming with the "repaired" primer. Thus the 3′-mismatch principle can be used to identify virtually any single point mutation within one or two PCR-SSP reactions,[8, 9] although it is important to note that primer-template mismatches other than the 3′-mismatch also have a bearing on the specificity of a primer (Figure 1).

The theoretical specificity of a PCR-SSP primer mix is derived from the intersection of both primer's specificities. For example, if a sense primer matches HLA-A*0101 and A*0102 and the antisense primer matches HLA-A*0101 and A*0103 and if PCR stringency is maintained, that primer mix will be specific for HLA-A*0101. To type an individual completely



There are seven different HLA-B sequences found between positions 259 and 272: all four bases can be found at position 272 coupled with a dimorphic motif at nucleotides 259 and 261.

Primer mix 73 uses a combination of primer 192 and 214 to identify many B*15 alleles, but it is mainly used for discriminating between the B*1501 like group of alleles and the B*1502 and B*1513 alleles. The mismatches at position 12 and 14 of primer 192 are sufficient to destabilise primer-template annealing in B*1502 and B*1503-positive individuals and thus allow discrimination between B*1501/3 and B*1502/13 groups. Thus for primer 192 the specificity depends on the nucleotide positions 259-272: this is termed the "significant length" of the primer [25]. In the case of primer 192 the significant length is 15. The significant lengths of the primers described in this chapter are given in Table 1.

Primers that utilise internal mismatches for their specificity must be carefully titrated and properly tested before use to prvent false-positive amplification of closely related alleles.

Figure 1. PCR-SSP reactions may use internal mismatches in primers for specificity as well as 3′-mismatches

at any given locus multiple PCR-SSP reactions are set up and subjected to PCR under identical conditions. The presence or absence of PCR amplification is detected by gel electrophoresis with visualization of the amplicons by ethidium bromide intercalating with the DNA fragment. An important feature of reliable SSP is that each individual reaction contains primers to amplify a so-called "housekeeping" gene[10] which detects possible PCR inhibition, thus acting as a positive control. Without this positive control it would be difficult to discriminate between a failed PCR reaction and a negative PCR reaction, making all homozygous results questionable.

Published PCR-SSP methods generally define a single locus[3, 10-22] or they may be, as described here, a combination of loci originally known as Phototyping.[23] The methods described herein are designed for medium resolution typing of HLA-A, B, C, DRB1, DRB3, DRB4, DRB5 and DQB1, which are updated from our previous publications.[21-23] The reactions described can be used as a whole set, or they can be broken up into various locus-specific units or they can be used individually as an adjunct to other PCR-SSP typing sets or other typing methods.

Specimens

DNA is usually obtained from EDTA or sodium citrate anticoagulated venous blood samples. It is strongly recommended that heparin is not used as an anticoagulant, since heparin interferes with *Taq* polymerase activity.[24] A rapid DNA preparation procedure is given in the methods section (See also Section V.A.1, this manual). Using the method given below, DNA may be extracted from old (up to 2 months) blood, clotted blood, or frozen blood samples. However, the best results are obtained from 1 to 5ml of fresh whole blood, with the latter volume recommended when possible. This DNA preparation method can also be applied to crude spleen or lymph node extract for HLA typing of cadaver donors.

Reagents and Supplies

A. DNA Preparation

1. Red cell lysis buffer (RCLB).
   0.144 M ammonium chloride (NH_4Cl)
   1 mM sodium bicarbonate ($NaHCO_3$)
   Dissolve 15.4 g of NH_4Cl and 1.68 g of $NaHCO_3$ in two liters of ddH_2O.
2. Nuclear lysis buffer (NLB).
   10 mM Tris-HCl pH 8.2
   0.4 M Sodium chloride (NaCl)
   2 mM di-sodium EDTA pH 8.0 (Na_2EDTA)
   Dissolve 23.37 g of NaCl in 900 ml of distilled water. Add 10 ml 1 M Tris-HCl pH 8.2 and 10 ml Na_2EDTA pH 8.0 and make up to one liter with distilled water.
3. 10% w/v sodium dodecyl-sulfate (SDS) – In a fume hood, dissolve 100 g of SDS in one liter of ddH_2O. Store at approximately 20° C to prevent formation of precipitate.
4. NLB+SDS buffer – Combine 300 ml of NLB with 20 ml of 10% w/v SDS. Store at approximately 20° C to prevent formation of precipitate.
5. 95% Ethanol – Combine 950 ml of absolute ethanol and 50 ml ddH_2O.
6. 70% ethanol – Combine 700 ml of absolute ethanol and 300 ml ddH_2O.
7. 6 M NaCl – Dissolve with warming 350.64 g of sodium chloride in 800 ml of ddH_2O and then make up to one liter with ddH_2O. 6 M NaCl is a saturated solution so not all of the NaCl will go into solution.

B. Ingredients for Primer Mixes

1. Synthesize or purchase primers (from Table 1) resuspended in ddH_2O at a concentration of 2000 µg/ml and stored frozen until required.
2. Cresol red 6 mg/ml stock – Dissolve 3 g of cresol red sodium salt in 500 ml ddH_2O. Pass through a 0.22 µm filter and store frozen in 1 ml volumes.
3. Stock solutions of control primers – Autoclave 2 L of ddH_2O. When cold, add 1.5 µl/ml each of DRB1 control primers[10] along with 10 µl/ml of cresol red and filter through a 0.22 µm filter. The control stock must be tested (see methods) prior to freezing in aliquots.

C. PCR Ingredients

1. 25 mM magnesium chloride – Add 1 ml 1 M stock solution to 39 ml ddH_2O. Note that stock solutions have approximately a 6 months shelf life, so when a new bottle gets opened dispense in 1 ml volumes and freeze.
2. dNTP mix: Combine 0.4 ml of each dNTP (dCTP, dATP, dTTP, dGTP) from 100 mM stock.
3. 10x Base buffer:
   670 mM Tris-base pH 8.9
   166 mM Ammonium sulfate
   1%v/v Tween 20 (Polyoxyethylenesorbitan monolaurate)
   Dissolve 40.568 g Tris base in 400 ml dH_2O and adjust to pH 8.9 with conc. HCl. Dissolve 10.96 g of ammonium sulfate in the Tris solution. Filtered through a 0.22 µm filter into an autoclaved bottle. Add 5 ml of Tween and make up to 500 ml with ddH_2O. Store at -70° C.

4. TDMH buffer (200 ml recipe). This when combined with all other PCR ingredients gives a final $MgCl_2$ concentration of 1.9 mM.

33.3 ml	x10 base buffer
25.1 ml	25 mM fresh $MgCl_2$
140.4 ml	freshly autoclaved water
1.215 ml	dNTP mix (i.e., all 4 mixed together)

Test TDMH buffer (see methods) before freezing in 13.3 ml aliquots.

D. Setting Up PCR Reactions

1. Method is described in the methods section. The extra ingredient required is 5 units/µl *Taq* polymerase.

E. Gel Electrophoresis

1. Orange G loading buffer: Combine 300 ml of glycerol, 250 ml of 2x TBE, 550 ml distilled water and 0.25 g orange G. Store at 20° C.
2. 2x and 0.5x TBE buffer

   To make 2x TBE dissolve 216 g of Tris-base and 110 g of boric acid in 9 L of distilled water. Add 8 ml of 0.5 M EDTA pH 8.3 and make up to 10 L with ddH_2O.

   To make 0.5x TBE combine 750 ml of distilled water with 250 ml of 2x TBE.
3. 1% agarose (one liter).

   10 g Electrophoresis grade agarose.
   One liter 0.5x TBE
   10 µl of 10 mg/ml ethidium bromide.

   Dissolve, by heating in a microwave, 10 g of agarose in 400 ml 0.5x TBE. Top up to one liter with 0.5x TBE and add 10 ml of 10 mg/ml ethidium bromide solution. Agarose solutions can be stored at 50°C for up to one week.

Instruments/Equipment

A. Specialist Plastics

96-well PCR plates. e.g., Advanced Biotechnologies AB-0600.
96-well plate sealers. e.g., Costar 6524

B. Dispensing Equipment

96-well dispenser. e.g., Robbins Scientific Hydra-96.
8-channel electronic multi dispenser.

C. PCR Machines

Models with 96-well or 384-well block format (384-well blocks work well for small volume PCR in either 384 or 96-well format PCR plates).

e.g., Perkins Elmer 9600 or 9700 thermalcycler, Techne Phoenix PCR machine, or MJ Research PTC200 PCR machine.

D. Horizontal Gel Casting Forms with Combs Suitable for Multichannel Loading

30x25cm Horizontal gel rig. e.g., Flowgen. G3-0403
1 mm 26 well sample combs. e.g., Flowgen. G3-0416
Large 312 nm transilluminator. e.g., Flowgen. T7-0174

E. Gel Imaging Systems

There are many gel imaging systems available from film-based to digital imaging. Below are examples of each approach:

Polaroid MP4 land camera system.

The Polaroid Land Camera is the most widely used approach. It gives excellent results and the camera itself is inexpensive but the film is relatively expensive. Additionally, you will also need to use Polaroid Type 667 B&W film in conjunction with Wratten 2A and Wratten 22 filters with this system.

Kodak gel analysis system

Digital camera and software, gives excellent results and permits embedding gel image and subsequent analysis into a report. Camera and software are expensive, but film is not necessary; ideal for high throughput laboratories.

Methods

Any typing method devised based on known sequences is always out of date by the time the method is published. It should be noted that allele sequences might be deleted or corrected over time and that these changes can influence the expected results of your typing system. It is therefore recommended that pertinent Internet accessible databases such as the *HLA Informatics Site (http://www.anthonynolan.com/HIG/index.html)* or the *IMGT database (http://mercury.ebi.ac.*

Table 3
Alleles considered or omitted for Table 2 from Nomenclature report 1998 (see reference 26)

Omitted Alleles	Extra Alleles
A*02012	A*0231
A*24022	A*0232N
A*2419	A*2421
A*2420	A*2422
B*1806	A*6810
B*3916	B*0807
Cw*0307	B*0808N
Cw*1508	B*3528
DRB5*0110N	B*4806
	B*7804
	DRB1*0314

uk/imgt/hla/) are frequently consulted for such changes. A suitable computer program such as SSP Manager for updating primer mix specificities is highly recommended.[25] The majority of alleles considered for the primers in Table 1 and the primer mixes shown in Table 2 were taken from the 1998 Nomenclature Report.[26]

Table 3 shows the alleles omitted (due to the sequence not being available) and additional alleles considered since publication of the Nomenclature Report.

The methods described here were initially described for Phototyping.[23] For efficient SSP amplification without false positive amplifications the conditions need to be highly stringent, as it is theoretically possible for 3′-mismatch extension.[8, 9, 27] SSP stringency is multifactorial, relying on the concentration of all the PCR constituents such as target DNA, *Taq*, dNTPs, Tris and free magnesium. PCR stringency kinetics also relies on individual primer factors such as primer sequence, length and type of primer-template mismatches. The following methods should provide the reader with enough information to efficiently test and set up and the PCR-SSP system of your choice.

A. Design of PCR Primers and PCR Primer Mixes

Consistent design of PCR primers along with use of the most up-to-date sequence alignments are key features of successful and accurate PCR-SSP HLA typing. All primers are initially designed to have a primer-template annealing temperature of 60° C or 62° C based on the popular formula 2X (number of A and T bases) + 4X (number of G and C bases) = annealing temperature in ° C. Generally the higher the annealing temperature the less specific the primer is likely to be. Ideally primers should have an even ratio of G/C to A/T bases but this is not always possible to achieve, and in fact some primers work well in PCR-SSP with 100% G/C content. Where possible primers are designed with the specificity-dependent nucleotide on the terminal 3′- nucleotide but internal mismatches in a primer may also significantly contribute to a primer's specificity as shown in Figure 1.

The primers described here for the updated Phototyping set are shown in Table 1. Purchase or synthesize primers as de-salted oligonucleotides on a 25 OD (approximately 0.2 mM) scale and resuspend in sterile distilled water at a concentration of 2000 mg/ml. Store frozen until required. Generally, primers can also be left at 4° C for long periods. The primer combinations giving rise to the primer mixes are shown in Table 2.

B. General Information on PCR-SSP Using Phototyping Methods

The basic tenet of the Phototyping method is that multiple primer mixes consisting of water, cresol red, allele-specific and control-specific primers are synthesized tested and stored in 1 ml primer mix volumes. A typing set collected from these stored primer mixes is dispensed in 3 µl volumes under mineral oil in 96-well or 384-well PCR plates. Separate from the primer mixes, a PCR buffer (called TDMH) containing all the other ingredients of PCR is made up, aliquoted, and stored frozen awaiting the addition of DNA and *Taq* polymerase. DNA is then added to a predetermined volume of the TDMH and 5 µl of this mixture is added to each well of the PCR plate prior to PCR amplification and agarose gel electrophoresis. This method allows extreme flexibility in the design and incorporation of any new primer mixes.

One of the key factors in maintaining PCR stringency is the concentration of the primers used: the concentrations given in Table 2 are to be used as a guide only as the optimal concentrations should be determined empirically within individual laboratories.

C. DNA Extraction

Good quality DNA is paramount for successful PCR-SSP. Sodium citrate or EDTA anticoagulated blood is preferred to heparinized blood as heparin is a severe inhibitor of PCR and especially PCR-SSP.[24] If heparinized blood is the only source, then DNA extraction using the heparinase protocol described below should allow for satisfactory typing. The following method is modification of Miller's salting-out procedure,[28] in which the use of Proteinase K is omitted and a chloroform extraction phase is added. This yields large quantities of good quality DNA suitable for PCR-SSP in less than 30 minutes.

1. Centrifuge 5 ml of EDTA or ACD- A anticoagulated blood to produce a buffy coat. Aspirate the buffy coat into a 15 ml polypropylene tube. Add 10 ml of RCLB, invert several times and leave to stand for 5 minutes.
2. Centrifuge at 1000 g for 10 minutes. Pour off supernatant and gently rinse pellet in 2 ml of RCLB. The pellet should be white with a pink halo. If there is too much hemoglobin, resuspend the pellet in RCLB, agitate and centrifuge. When the pellet is homogeneously white it can be stored at -70°C or you can continue to the next step.
3. Resuspend pellet in 3 ml of NLB+SDS (warm NLB+SDS if precipitate visible). Add 1 ml of 6 M NaCl, vortex (precipitate should be visible). Add 2 ml of chloroform and shake until homogenous milky solution is seen. Centrifuge for 10 minutes at 1000 g.
4. Aspirate the DNA (top phase) into a 20 ml tube. If the DNA phase is not clear in appearance transfer to a clean polypropylene tube and repeat the chloroform extraction step. Be careful not to suck up any protein from the interface.
5. Add two volumes of 95% ethanol, gently rock until all of the DNA is precipitated. Centrifuge for 5 minutes at 700 g and resuspend in 70% ethanol, centrifuge and repeat this washing step.
6. Transfer the DNA precipitate into a sterile 0.5 ml microcentrifuge tube, pellet the DNA, and remove the excess ethanol either by centrifugal evaporation, lyophilization, or allowing it to dry on the bench. Resuspend the DNA in 300 µl of sterile ddH_2O. From 5 ml of blood you can expect to obtain DNA concentrations in the range of 0.2 to 1.0 mg/ml. Any DNA sample with a concentration within the 0.2 to 1.0 mg/ml range is suitable for PCR-SSP without modification of the DNA volume to be added (see setting up PCR-SSP section).

D. Dispensing Primer Mixes

Tested primer mixes (see notes on batch testing PCR-SSP reagents) should be dispensed in 1 ml volumes in 1 ml straight tubes which are suitable for placing in standard 96-well format in a 96-well rack. These tubes and racks are suitable for use both with 8/12 channel hand-held electronic multi-dispensing pipettes and also with 96-well robotic dispensers such as the Robbins Hydra. Using a 12 channel electronic dispensing pipette add 10 µl of mineral oil to 96 well PCR plates. Dispense 3 µl of each primer mix into the appropriate wells of the PCR plates using the Robbins Hydra dispenser. Completed trays may be stored for 6-12 months at -30° C, preferably in sealed bags or with individual plate sealers.

E. Setting up PCR-SSP Using TDMH Buffer

Thaw out plate(s) containing the primer mixes. Thaw out a 13.3 ml aliquot of TDMH and add 64 µl of 5 units/µl *Taq* polymerase. This mixture will keep at 4° C for at least one week. Count how many individual PCR-SSP reactions are required for each individual DNA sample (protocol given here is for 192 reactions). For each 3 µl primer mix, 5 µl of TDMH/DNA/*Taq* mixture is added. It is important for maintenance of the $MgCl_2$ concentration that the ratio of TDMH to all other PCR ingredients is 1:0.6. Thus, for 192 reactions add 20 µl of DNA to 1184 µl of TDMH/*Taq* mix. Vortex briefly and pour mixture into a disposable trough. Using an 8-channel electronic multidispensing pipette and draw up the appropriate volume. Dispense 5 µl of DNA/TDMH/*Taq* mixture to 8 wells at a time; keep the pipette tip at the top edge of the mineral oil meniscus and allow the mixture to roll off the tip and through the mineral oil. Do not allow the tips to touch the primer mix otherwise carry-over, and consequently false-positive amplifications may occur. On addition of the TDMH mixture to the primer mixes the cresol red will change color from yellow to purple. When the tray is complete, seal with a fresh tray sealer, centrifuge briefly (200 g for 5 seconds) to ensure all PCR reactions are mixed and submerged below the oil (vortex mixing of completed plates is not recommended).

F. Setting up PCR-SSP Using Heparin-contaminated DNA

Make a 0.2 unit/µl solution of heparinase II by adding 50 µl of ddH_2O to a 10 unit vial. Add 5 µl heparinase per 15 µl DNA, agitate and incubate for 90 minutes at 37°C. Add to TDMH mixture as normal. Heparinase activity is destroyed by freeze-thawing.

G. PCR Amplification Program

This program is suitable for the majority of PCR machines and takes about 1.5 hours to run:

96° C for 60 seconds.
96° C for 20 sec, 70° C for 45 sec and 72° C for 25 sec (x 5 cycles)
96° C for 25 sec, 65° C for 50 sec, 72° C for 30 sec (x 21 cycles)
96° C for 30 sec, 55° C for 60 sec, 72° C for 90 sec (x 4 cycles)

Prior to termination of the program, cool by ramping to 20° C for 30 sec

Some thermoplastics used for PCR are not an exact fit for every PCR machine and consequently accurate heat transfer to the PCR reaction may be effected. Too ensure correct thermodynamics we dip the PCR vessels into a little light paraffin oil, and blot excess on tissues before placing in PCR machines.

Note: Apply firm and even pressure to the top surface PCR vessels during thermocycling. Preferably use a heated lid.

H. Electrophoresis

Use large electrophoresis tanks utilizing gel trays accommodating gel combs with teeth spatially separated for use with multichannel pipettes. Pour 400 ml of 1% agarose into the taped off gel tray and insert the combs, allow 20 minutes to set. Fill electrophoresis tank with 2.2 L of 0.5x TBE (can be left in tank and re-used at least 15 times). Remove tape and combs and submerge gel tray in tank. Using a multichannel Hamilton syringe, add 5 µl of orange G loading buffer. Using a multichannel pipette load 18 µl of 8 or 12 PCR reactions at a time to the gel (depending on tray layout). Electrophorese for 20 minutes at 200V or until the orange G can be seen to have traveled approximately 3 cm.

I. Gel Photography

PCR amplicons are visualized via 312 nm UV transillumination. Results are recorded by gel photography using either Polaroid photography in conjunction with Wratten 22 and 2a filters or any other suitable imaging system as described above. Using the Polaroid system, a shutter speed of 2 and aperture of f5.6 with Polaroid type 667 film is suitable for gel photography.

To facilitate identification of positive PCR reactions it is recommended that the electrophoresis lanes be labeled by using an overhead projector (OHP) acetate with the lane numbers printed in the correct spatial orientation. The OHP acetate is laid over the gel in the correct position prior to photography. It is recommended that the OHP is laminated (to prevent wear) and that windows between one row of number and another (where the PCR amplicons appear) are cut out to reduce interference from the plastic fluorescing in UV light.

J. Interpretation of Results

PCR-SSP interpretation of HLA genotypes is relatively easy, and generally results can be interpreted with little or no prior experience. Each PCR-SSP reaction is deemed to have worked if the control amplification is present. Positive allele-specific amplifications are identified by the presence of a correct sized PCR allele-specific amplicon, relative to the known molecular size markers, while absence of an allele-specific amplicon implies absence of the alleles identified in a given primer mix. If a reaction has neither control nor allele-specific amplicons the reaction has failed and is deemed "not tested". The alleles that would have been amplified in this reaction are therefore also not tested. Fortunately, many alleles are amplified in more than one reaction so sporadic PCR failures do not often affect full assignation of a genotype. If all of the reactions have failed then the whole result is not tested and must be repeated (see trouble shooting in the *Notes* section). Alleles are assigned by identifying the pattern of positive and negative reactions and interpreting these with reference to the information given in Table 2.

Calibration

The PCR-SSP methods described here are relatively forgiving of pipetting errors. However, ASHI Standards dictate that pipettes be checked frequently for accuracy. ASHI Standards also state that PCR machines should be checked monthly for block uniformity by amplifying 96 identical reactions in a 96-well plate and checking for even amplification of both controls and alleles. If the thermalcycler fails to obtain even amplification in all 96 wells, the block should be repaired or replaced. In those laboratories with multiple machines, it is a wise practice to keep a log of which PCR machine is used for individual typing results to better monitor for cycler failure.

Quality Control

It is essential that all the PCR reagents and consumables are tested for efficiency before routine typing commences. The PCR buffer and its key ingredients such as dNTP's, magnesium chloride and *Taq* polymerase should be tested for optimal concentration. Some *Taq* polymerases are more efficient than others are, so it is important to find the optimal concentration for your reagent. The ratio of dNTP to magnesium chloride is critical and it is advisable to freeze aliquots of magnesium chloride solution rather than store on the shelf, as the reagent will deteriorate over time.

All primer mixes should be batch tested and stored frozen in suitably sized aliquots. Where possible primer mixes should be tested with both positive and negative samples as well as a no-DNA control (or open tube) for PCR contamination. Periodically all PCR reagents should be tested for contamination: if DNA or PCR amplicon contamination is suspected the reagent must be discarded.

To avoid PCR contamination it is recommended that DNA preparation and pre-PCR steps be performed in a different room than that of post-PCR manipulation. No laboratory equipment should be removed from the post-PCR room to the pre-PCR room and vice versa. Obviously gloves and other personal protective equipment used in post-PCR steps should be removed upon leaving the post-PCR room. If bench-swab PCR contamination tests are performed in the pre-PCR rooms, you must make sure that the swab method has a suitable control for PCR inhibitors.

Finally, the laboratory should ensure that the PCR plates in use fit snuggly into the wells of the PCR machine as not all PCR plates and PCR blocks are compatible. Ill-fitting trays will result in uneven amplification throughout the tray.

Notes on Batch Testing PCR-SSP Reagents

A. Testing Control Solutions

Getting the right concentration of control primers in the stock control solutions is of vital importance as these solutions are the basis for all the primer mixes. The concentration of primers must be not so high that the allele-specific amplicon is out-competed for by the controls. On the other hand, if the concentrations of control primers are too low then the control amplicons will be difficult or impossible to visualize, leaving many primer mixes to appear non-functional. To establish a good working concentration, titrate the control primers (suggested titration: 5, 2.5, 1.25 µl/ml) in the presence of a constant concentration of a pair of allele-specific primers. Test this titration against some DNA samples of varying quality and of varying genotype (some positive and some negative for the allele-specific primers). The optimal concentration of control primers is found when the control amplicon does not out compete the allele-specific amplicon in HLA allele-positive reactions and yet is present in all allele-negative samples.

B. Testing Allele-specific Primers

The majority of primer mixes will function well using the recommended concentrations given in Table 2. However, there is some variability in amplification efficiencies between batches of oligonucleotides and primer mixes so that every new synthesis of primer mixes must be properly tested as optimal primer concentrations will fluctuate from batch to batch. If large volumes (20-50 ml) of primer mixes are being synthesized for the first time, it is advisable to test the recommended concentrations first by making up 0.5 ml aliquots and testing on appropriate control samples. An ideal primer mix should produce allele-specific amplicons that are easily visible in all expected positive samples and clearly negative in expected negative samples: if possible, test some DNA of poor quality as well as normal DNA to ensure a robust primer mix is obtained. If a primer mix is weak or negative with expected positive DNA samples, increasing the concentration of primers usually improves results. Similarly, false-positive amplifications can be removed by titrating either one (asymmetric titration) or both of the primers. Sense primers with the 3'- end at position 272 (for example primer 192) all utilize internal mismatches at positions 12 and 14 of the primer. Primer mixes involving these primers require careful testing and subsequent titration of the sense primer to avoid weak amplification of similar sequences. For example, primer mix 73 is designed to amplify B*1501 and some other B*15 alleles but not B*1502. Because the primer 192 is only mismatched internally between B*1501 and B*1502 it is possible B*1502 might be amplified in this mix, requiring the mix be tested with B*1502 as well as B*1501 before being put into general use.

Initially, some primer mixes (especially primer mixes 152 and 76) fail to produce allele-specific and control-specific amplicons, instead they produce primer-dimers. These primers also need careful reduction of the allele-specific primers to a point where they amplify alleles but do not go to primer-dimer formation.

Some primer mixes only detect very rare alleles (such as HLA-A*4301 or B*4802) and are consequently difficult to positively test for unless reference DNA samples are available. If reference samples are not available, test the individual primers in different combinations to estimate the optimal concentrations. For example, if the HLA-A*4301 primer mix (PM12) is not positively tested, the sense primer (primer 174) which is specific for A*2901-3 as well as A*4301, could be tested in conjunction with another antisense primer that also detects A*2901-3. Similarly the antisense primer (primer 298) could be tested and the optimal concentration of primers could be extrapolated to the A*4301-specific primer mix. If a primer can not be tested in this way because it's sequence is unique to a rare allele it is best to use it at the highest possible concentration that does not produce false-positives.

C. Testing TDMH

Each batch of TDMH should be tested in comparison with the previous batch before general use. It is best to test several different DNA samples of different phenotypes to ensure that the buffer is efficient for most primer mixes. The most common defect found when testing TDMH is that the control bands appear strong but the allele bands appear weak, or even non-existent. This is commonly caused by an imbalance in the $MgCl_2$ to dNTP ratio, i.e., either too high a concentration of $MgCl_2$ or too low a concentration of dNTP's has been used. It is thought that, since dNTP's efficiently chelate $MgCl_2$, an excess of dNTP's sequesters free $MgCl_2$ and thus deprives *Taq* of the magnesium that it requires as a co-factor.

Troubleshooting

1. All reactions have failed (no allele, no control-specific amplicons).

This may be due to either the poor quality of or insufficient amount of DNA: Test another DNA sample previously shown to work with the test reagents and PCR machine. If poor quality DNA is suspected, using less DNA with 50% more *Taq* may work. If gel electrophoresis reveals an adequate amount of DNA to be present in the mixture, then heparin or protein contamination could have caused the negative amplification. If heparin contamination is suspected, use the heparinase II protocol to inactive it.

Protein contamination may be dealt with by re-extracting the DNA using a modified salting out procedure. A 20% v/v solution of 6 M NaCl is added to the remaining DNA, along with an equal volume of chloroform and mixed by vortex. The solution is centrifuged at high speed in a microfuge for 5 minutes and the aqueous DNA phase is extracted and ethanol precipitated as above (See also Section V.A.1, this manual).

When DNA samples shown to previously work start failing it is possible that one of the PCR ingredients is faulty or that the DNA sample has degraded over time. For this reason, it is good practice to always keep a batch of working frozen stock ingredients so that trouble shooting can be made easier. Fluctuations in amplification can also be due to variation in *Taq* supply. Ensure that the *Taq* has been titrated for optimal performance, checked periodically for robustness of reactions, and stored in frozen aliquots.

2. Generally weak reactions.

Weak reactions are usually due to insufficient or poor quality DNA. Try adding more DNA and repeat the amplification. If this does not work see the above section on failures.

Incorrectly made buffer or poor/dilute *Taq*. Remake buffer or try increasing *Taq* concentrations. Some laboratories use twice or three times the *Taq* concentration suggested in this procedure. It is up to each laboratory to determine the vendor of choice for and validate the optimal concentration of *Taq* for their reagents and thermalcycler.

Inefficient thermalcyclers: not all PCR machines work well for the PCR-SSP protocol described in this section. If reactions are always weak, try increasing some of the PCR program sections (times and temperatures) or try lower annealing temperatures at the start of the program (68° C instead of 70° C).

3. Too many allele-specific amplicons in one locus.

Possible new allele: Try to confirm by PCR mapping techniques[29, 30] or by using another molecular method such as sequencing or SSOP.

Sample contaminated with another DNA sample: Most such contamination would yield extra bands at all loci tested but it is possible to get a combination of alleles in two samples so that the contamination was only noticed at one locus. Most accidental contamination involves small amounts of extraneous DNA being introduced to a larger amount so that the contaminating bands are typically weak but consistent.

Sample contaminated with a locus-specific amplicon from another part of the laboratory: try to minimize contamination by maintaining good laboratory techniques using spatial separation of pre and post-amplification are as well as dedicated utensils and personal protective equipment.

Incorrectly made up or contaminated primer mix: Retest suspected primer mix and, if found faulty, re-synthesize or repurchase them.

4. Too many allele-specific amplicons in all loci.

Thermalcycler error: If the PCR program is interrupted and re-started (especially at the early stages) multiple bands are seen due to the low stringency PCR induced. For that reason it is best to use a PCR machine that gives error messages when programs have been interrupted.

Sample or PCR buffer contamination: Remake solutions if contaminated.

5. No allele-specific amplicons at one locus.

Homozygous example of a new allele not detected by the given PCR reactions. This is unlikely.

Incorrectly made primer mixes: ensure all primer mixes are tested before use.

Incorrect buffer mixture: If the dNTP to $MgCl_2$ ratio is incorrect it can effect one locus more than another so that it appears as if there are no alleles at one particular locus. The HLA-B locus, especially the primer mixes specific for B*44, B*08, B*49 and B*51, are most susceptible to this phenomenon. This possibly may be due to these alleles having a higher G/C ratio in the introns than other alleles and thus *Taq* may have difficulty in amplifying these regions. Classically false-negative allele amplifications due to incorrect $MgCl_2$ concentrations are associated with much stronger control amplicons and much weaker than normal allele-specific amplicons.

6. Individual reaction failure.

Approximately 0.5-1% of PCR-SSP reactions spontaneously fail for no apparent reason. Possible causes include incomplete PCR reaction, PCR inhibitory contaminant in an individual well, or failure of individual PCR vessels. If a reaction has failed and no primer or primer-dimer is visible on the gel, it is likely that either the agarose well was incompletely formed or the reaction mixture was not loaded into the gel properly.

7. Allele-specific bands present but no controls.

Degraded DNA may produce only small amplicons such as the allele-specific amplicons but not larger amplicons such as the control amplicon.

Insufficient PCR extension time: try increasing the time the PCR program spends at the extension temperature (72° C)

PCR machine needs re-calibration.

Concentration of control primers is too low.

8. Control-specific amplicons but no alleles.

Magnesium concentration too high: re-calibrate TDMH.

PCR program is inefficient. Try different PCR programs.

Poor fit of PCR tubes/plate into PCR block: The bottom of the PCR vessel must be in direct contact with the PCR block otherwise the correct temperature will not be uniformly applied to the PCR reactions. If the tube or plate fit is suspect, dip the vessels in a little light paraffin oil to coat the exterior of the vessel before placing in the PCR block.

Insufficient pressure from above: If pressure is not applied to the PCR plate the plate may lift out of the block slightly or the thermoseal may peel off. In either case you end up with different PCR thermodynamics which can produce allele drop-out.

Primer mix inefficient at PCR temperature: Some primer mixes do not perform as well as others under the same PCR conditions and this is frequently due to the primer mix being required to work at an inappropriate temperature. Check the primer mix at different PCR temperatures to see if the primer mix works more efficiently at a different temperature. This can be done using several thermalcyclers, or more conveniently with one that has a gradient block such as the Eppendorf Mastercycler Gradient PCR machine. If one of the primer mixes works at a lower temperature than that required by the rest of the primer mixes in the set, one or both of the primers will have to be lengthened by one or two bases to increase the primer(s) annealing temperature. If a primer pair works at a hotter temperature, it indicates there is a structural problem with one of the primers (such as primer sequence tending to form hairpin loops. It is recommended that those primer mixes with inherent structural problems be redesigned or that the primer concentration is radically increased.

9. Part of the typing has worked well, but the remainder has failed.

Thermalcycler failure. This is a common problem when a PCR machine is used intensively. That is why it is necessary to test block uniformity on a regular basis by amplifying 96 identical reactions in one plate. If a problem does exist, a PCR service engineer or the manufacturer should be contacted.

PCR plate not placed in machine properly.

Uneven pressure applied during PCR.

Gel artifact caused by insufficient ethidium bromide.

Limitations of the Procedures

One disadvantage of relying on molecular typing methods based solely on existing sequence information is that new allelic variants may not be detected at all, or they may not be discriminated from a known allele. To avoid missing new allelic variants when using just PCR-SSP or SSOP, a policy of using large numbers of primers or probes can be adopted. This has the dual advantage of potentially detecting novel alleles and enhancing the resolution of the typing system. However, even extensive SSP or SSOP typing systems can not discriminate between normally expressed alleles and expression variants on their own, unless chance dictates that the novel mutation disrupts the annealing of a primer or probe to target DNA. Thus, null alleles and those expressed at a low level are normally only detected by a combination of serological and molecular methods. Once the mutation giving rise to null alleles is discovered, PCR-SSP reactions can be devised to identify the polymorphism. Some mutations giving rise to null alleles are likely recur in other alleles: an example of this is the cytosine insertion in a row of cytosines anywhere between positions 620 and 627 which is responsible for the lack of expression of A*2411N,[31] A*01014N,[32] and B*5111N. By designing PCR-SSP primers to detect this site of recurring mutation it is possible to not only detect specific null alleles but also to screen for other null alleles with this insertion.[33]

Updating the Typing System

The ever-increasing number of HLA alleles makes updating large PCR-SSP typing systems very difficult. Each new allele must be cross-referenced with the two (or more) *cis*-located polymorphisms identified in each primer mix and internal mismatches with primer lengths must also be considered. This is a difficult, time consuming task, which frequently leads to errors being made. To facilitate updating various primer mixes sets, the reader is encouraged to use a computer program such as "PCR-SSP Manager".[25] This program allows all new HLA sequences to be aligned. Once a new allele is inserted, the specificities of all the primers and consequently the primer mixes are adjusted and the new updated specificity list for a tray of reactions is produced. Furthermore, the program can assist the investigator developing new primer mixes by suggesting new primer mixes to sort out the inevitable conundrums created by new alleles. Added to this are various interpretation facilities that make the program an invaluable tool for anyone using PCR-SSP for HLA typing. This program is available on request from the Oxford Transplant Immunology Laboratory (e-mail at *mbunce@hgmp.mrc.ac.uk*).

References

1. Jordan F, McWhinnie AJ, Turner S, Gavira N, Calvert AA, Cleaver SA, Holman RH, Goldman JM, Madrigal JA. Comparison of HLA-DRB1 typing by DNA-RFLP, PCR-SSO and PCR-SSP methods and their application in providing matched unrelated donors for bone marrow transplantation. *Tissue Antigens* 1995; 45 (2): 103-10.
2. Mytilineos J, Lempert M, Middleton D, Williams F, Cullen C, Scheren S, Opelz G. HLA class I typing of 215 "HLA-A, B, -DR zero mismatched" kidney transplants. *Tissue Antigens* 1997; 50 (4): 355-358.
3. Bunce M, Barnardo MCNM, Procter J, Marsh SGE, Vilches C, Welsh KI. High resolution HLA-C typing by PCR-SSP: identification of allelic frequencies and linkage disequilibria in 604 unrelated random UK Caucasoids and a comparison with serology. *Tissue Antigens* 1996; 48: 680-691.
4. Lorentzen DF, Iwanaga KK, Meuer KJ, Moritz TL, Watkins DI. A 25% error rate in serologic typing of HLA-B homozygotes. *Tissue Antigens* 1997; 50 (4): 359-365.
5. Yu N, Ohashi M, Alosco S, Granja C, Salazar M, Hegfland J, Yunis E. Accurate typing of HLA-A antigens and analysis of serological deficiencies. *Tissue Antigens* 1997; 50 (4): 380-386.
6. Chien A, Edgar DB, Trela JM. Deoxyribonucleic acid polymerase from the extreme thermophile Thermus aquaticus. *J Bacteriol* 1976; 127 (3): 1550-7.
7. Tindall KR, Kunkel TA. Fidelity of DNA synthesis by the Thermus aquaticus DNA polymerase. *Biochemistry* 1988; 27 (16): 6008-13.
8. Wu DY, Ugozzoli L, Pal BK, Wallace RB. Allele-specific enzymatic amplification of beta-globin genomic DNA for diagnosis of sickle cell anemia. *Proc Natl Acad Sci U S A* 1989; 86 (8): 2757-60.
9. Newton CR, Graham A, Heptinstall LE, Powell SJ, Summers C, Kalsheker N, Smith JC, Markham AF. Analysis of any point mutation in DNA. The amplification refractory mutation system (ARMS). *Nucleic Acids Res* 1989; 17 (7): 2503-16.
10. Olerup O, Zetterquist H. HLA-DR typing by PCR amplification with sequence-specific primers (PCR-SSP) in 2 hours: an alternative to serological DR typing in clinical practice including donor-recipient matching in cadaveric transplantation [see comments]. *Tissue Antigens* 1992; 39 (5): 225-35.
11. Bunce M, Taylor CJ, Welsh KI. Rapid HLA-DQB typing by eight polymerase chain reaction amplifications with sequence-specific primers (PCR-SSP). *Hum Immunol* 1993; 37 (4): 201-6.

12. Olerup O, Aldener A, Fogdell A. HLA-DQB1 and -DQA1 typing by PCR amplification with sequence-specific primers (PCR-SSP) in 2 hours. *Tissue Antigens* 1993; 41 (3): 119-34.
13. Knipper AJ, Hinney A, Schuch B, Enczmann J, Uhrberg M, Wernet P. Selection of unrelated bone marrow donors by PCR-SSP typing and subsequent nonradioactive sequence-based typing for HLA DRB1/3/4/5, DQB1, and DPB1 alleles. *Tissue Antigens* 1994; 44 (5): 275-84.
14. Browning MJ, Krausa P, Rowan A, Bicknell DC, Bodmer JG, Bodmer WF. Tissue typing the HLA-A locus from genomic DNA by sequence-specific PCR: comparison of HLA genotype and surface expression on colorectal tumor cell lines. *Proc Natl Acad Sci U S A* 1993; 90 (7): 2842-5.
15. Krausa P, Bodmer JG, Browning MJ. Defining the common subtypes of HLA A9, A10, A28 and A19 by use of ARMS/PCR. *Tissue Antigens* 1993; 42 (2): 91-9.
16. Krausa P, Browning MJ. A comprehensive PCR-SSP typing system for identification of HLA-A locus alleles. *Tissue Antigens* 1996; 47: 237-244.
17. Sadler AM, Petronzelli F, Krausa P, Marsh SG, Guttridge MG, Browning MJ, Bodmer JG. Low-resolution DNA typing for HLA-B using sequence-specific primers in allele- or group-specific ARMS/PCR. *Tissue Antigens* 1994; 44 (3): 148-54.
18. Savelkoul PH, de Bruyn-Geraets DP, van den Berg-Loonen EM. High resolution HLA-DRB1 SSP typing for cadaveric donor transplantation. *Tissue Antigens* 1995; 45 (1): 41-8.
19. Bunce M, Welsh KI. Rapid DNA typing for HLA-C using sequence-specific primers (PCR-SSP): identification of serological and non-serologically defined HLA-C alleles including several new alleles. *Tissue Antigens* 1994; 43 (1): 7-17.
20. Bunce M, Barnardo MC, Welsh KI. Improvements in HLA-C typing using sequence-specific primers (PCR-SSP) including definition of HLA-Cw9 and Cw10 and a new allele HLA-"Cw7/8v". *Tissue Antigens* 1994; 44 (3): 200-3.
21. Bunce M, Fanning GC, Welsh KI. Comprehensive, serologically equivalent DNA typing for HLA-B by PCR using sequence-specific primers (PCR-SSP). *Tissue Antigens* 1995; 45 (2): 81-90.
22. Gilchrist FC, Bunce M, Lympany PA, Welsh KI, du Bois RM. Comprehensive HLA-DP typing using polymerase chain reaction with sequence-specific primers and 95 sequence-specific primer mixes. *Tissue Antigens* 1998; 51 (1): 51-61.
23. Bunce M, O'Neill CM, Barnardo MCNM, Krausa P, Browning MJ, Morris PJ, Welsh KI. Phototyping: Comprehensive DNA typing for HLA-A, B, C, DRB1, DRB3, DRB4, DRB5 & DQB1 by PCR with 144 primer mixes utilising sequence-specific primers (PCR-SSP). *Tissue Antigens* 1995; 46 (5): 355-367.
24. Satsangi J, Jewell DP, Welsh K, Bunce M, Bell JI. Effect of heparin on polymerase chain reaction [letter]. Lancet 1994; 343 (8911): 1509-10.
25. Bunce M, Barnardo MCNM, Welsh KI. The PCR-SSP Manager Computer Program: A tool for maintaining sequence alignments and automatically updating the specificities of PCR-SSP primers and primer mixes. *Tissue Antigens* 1998; 52 (2): 159-175.
26. Bodmer JG, Marsh SGE, Albert ED, Bodmer WF, Bontrop RE, Dupont B, Erlich HA, Hansen JA, Mach B, Mayr WR, Parham P, Petersdorf EW, Sasazuki T, Schreuder GMT, Strominger JL, Svejgaard A, Terasaki PI. Nomenclature for factors of the HLA system, 1998. *Tissue Antigens* 1999; 53 (4, part II): 407-446.
27. Kwok S, Kellogg DE, McKinney N, Spasic D, Goda L, Levenson C, Sninsky JJ. Effects of primer-template mismatches on the polymerase chain reaction: human immunodeficiency virus type 1 model studies. *Nucleic Acids Res* 1990; 18 (4): 999-1005.
28. Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res* 1988; 16 (3): 1215.
29. Vilches C, Bunce M, de Pablo R, Herrero MJ, Kreisler M. Anchored PCR cloning of the novel HLA-Cw*0704 allele detected by PCR-SSP. *Tissue Antigens* 1995; 46 (1): 19-23.
30. Krausa P, Barouch D, Bodmer JG, Browning MJ. Rapid characterization of HLA class I alleles by gene mapping using ARMS PCR. *Eur J Immunogenet* 1995; 22 (3): 283-7.
31. Magor KE, Taylor EJ, Shen SY, Martinez-Naves E, Valiante NM, Wells RS, Gumperz JE, Adams EJ, Little A-M, Williams F, Middleton D, Gao X, McCluskey J, Parham P, Lienert-Weidenbach K. Natural inactivation of a common HLA allele (A*2402) has occurred on at least three separate occasions. *The Journal of Immunology* 1997; 158: 5242-5250.
32. Laforet M, Froelich N, Parissiadis A, Pfeiffer B, Schell A, Faller B, Woehl-Jaegle M-L, Cazenave J-P, Tongio M-M. A nucleotide insertion in exon 4 is responsible for the absence of expression of an HLA-A*01 allele. *Tissue Antigens* 1997; 50 (4): 347-350.
33. Bunce M, Procter J, Welsh KI. A DNA based detection and screening system for identifying HLA class I expression variants by sequence-specific primers. *Tissue Antigens* 1999; 53 (5): 498-506.

Table 1a. Class II Primers

No.	Position	SP	O	Sequence 5'-3'
36	22-43	14	S	TTgTggCAgCTTAAgTTTgAAT
41	20-38	14	S	TCCTgTggCAgCCTAAgAg
44	88-109	14	S	TACTTCCATAACCAggAggAgA
46	60-77	14	S	gACggAgCgggTgCggTA
47	17-38	14	S	gTTTCTTggAgCAggTTAAACA
48	21-40	14	S	CCTgTggCAgggTAAgTATA
50	26-46	14	S	AgTACTCTACgggTgAgTgTT
52	17-39	14	S	gTTTCTTgAAgCAggATAAgTTT
53	73-91	14	S	CggTTgCTggAAAgACgCg
61	17-38	14	S	gTTTCTTgCAgCAggATAAgTA
68	17-38	14	S	gTTTCTTggAgTACTCTACgTC
69	18-38	14	S	TTTCTTggAgCTgCgTAAgTC
70	17-38	14	S	gTTTCTTggAgCTgCTTAAgTC
76	135-152	14	S	ggAgTACCgggCggTgAg
77	44-63	14	S	gCTACTTCACCAACgggACC
79	61-77	14	S	ACggAgCgCgTgCgggg
82	70-89	14	S	gTgCgTCTTgTgAgCAgAAg
181	60-77	14	S	gACggAgCgCgTgCgTTA
263	75-94	14	S	gTTCCTggACAgATACTTCC
270	-20 to +1	14	S	gATCgTTCgTgTCCCCACAA
273	19-38	14	S	TTCTTggAgTACTCTACggg
283	-20 to +1	14	S	gATCgTTCgTgTCCCCACAg
347	18-38	14	S	TTTCgTgCTCCAgTTTAAggC
348	127-144	14	S	gACgTgggggTgTACCgC
349	63-81	14	S	ggAgCgCgTgCgTCTTgTA
353	70-89	14	S	gTgCgTCTTgTgACCAgATA
650	71-89	14	S	TgCggTTCCTggACAgACA
1465	59-77	14	S	ggACggAgCgCgTgCgTCT
1630	158-174	14	S	TggggCggCCTgATgAg
1631	154-170	14	S	gAgCTggggCggCCTgC
1634	81-102	14	S	ggACAgATACTTCTATAACCAA
1674	14-32	10	S	CACgTTTCTTggAgCTgTg
2489	87-109	10	S	ATACTTCCATAACCAggAggAgA
37	257-276	10	AS	CTgCACTgTgAAgCTCTCAC
38	257-276	10	AS	CTgCACTgTgAAgCTCTCCA
39	199-216	10	AS	CCgCCTCTgCTCCAggAg
40	199-217	12	AS	CCCgCTCgTCTTCCAggAT
42	199-216	14	AS	CCgCgCCTgCTCCAggAT
49	232-252	14	AS	CCCgTAgTTgTgTCTgCACAC
51	220-239	14	AS	CTgCAgTAggTgTCCACCAg
54	173-192	14	AS	CTggCTgTTCCAgTACTCCT
55	254-273	14	AS	CACTgTgAAgCTCTCCACAg
58	221-240	13	AS	TCTgCAATAggTgTCCACCT
78	231-251	14	AS	TggTAgTTgTgTCTgCATACg
102	169-187	14	AS	TgTTCCAgTACTCggCgCT
104	199-217	12	AS	CCCgCCTgTCTTCCAggAA
107	170-188	14	AS	CTgTTCCAgTgCTCCgCAg
111	250-266	14	AS	CCgCggAACgCCACCTC
112	250-267	14	AS	CgTgCggAgCTCCAACTg
151	231-251	14	AS	CCgTAgTTgTgTCTgCAgTAA
152	250-267	14	AS	CCCgCggTACgCCACCTC
252	211-226	14	AS	CCACCgCggCCCgCgC
255	212-228	9	AS	gTCCACCCggCCCCgCT
256	170-188	14	AS	CTgTTCCAggACTCggCgA
258	208-224	8	AS	ACCgCggCCCgCCTgTC
259	211-227	14	AS	TCCACCgCggCCCgCTC
268	220-239	11	AS	CTgCAgTAATTgTCCACCCg
314	126-144	14	AS	CTggTACTCCCCCAggTCA
350	220-238	14	AS	TgCACACCgTgTCCAACTC
351	221-238	11	AS	TgCACACCCTgTCCACCg
485	211-227	14	AS	TCCACCgCggCCCgCTT
491	140-156	14	AS	CTCCgTCACCgCCCggT
492	171-189	14	AS	gCTgTTCCAgTACTCAgCg
498	212-228	14	AS	gTCCACCgCggCCCgCT
500	221-238	14	AS	TgCAgTAggTgTCCACCT
501	221-239	14	AS	CTgCAgTAggTgTCCACCg
651	199-216	14	AS	CCgCgCCTgCTCCAggAA
831	170-191	14	AS	TggCTgTTCCAgTACTCggCgg
866	130-147	10	AS	CgCCTggTACTCCCCCAg
1466	170-189	14	AS	gCTgTTCCAgTACTCggCgT
1635	199-217	12	AS	CCCgCCTgTCTTCCAggAT
2510	169-190	14	AS	ggCTgTTCCAgTACTCggCATC

The No. column is the primer identification. The Position column refers to the annealing position of the primer. The SP column refers to the significant places used for individual primers (see text on primer design). The O column desinates whether the primer is sense (S) or antisense (AS).

Table 1b. Class I Primers

No.	Position	SP	O	Sequence 5'-3'	No.	Position	SP	O	Sequence 5'-3'
1	263-282	12	S	CACggAATgTgAAggCCCAC	166	302-318	14	AS	gCgCAggTTCCgCAggC
4	284-302	14	S	CACAggCTgACCgAgTgAg	167	559-576	14	AS	gAgCCACTCCACgCACCg
130	402-419	14	S	CCgCgggTATgACCAgTC	168	559-576	10	AS	gAgCCACTCCACgCACgT
159	325-343	14	S	TACAACCAgAgCgAggCCA	170	538-556	14	AS	CCTCCAggTAggCTCTCTg
160	326-343	14	S	ACAACCAgAgCgAggCCg	171	391-407	14	AS	CCgCggAggAAgCgCCA
165	158-176	14	S	ACgACACgCAgTTCgTgCA	183	368-384	14	AS	CCCCAggTCgCAgCCAg
173	78-98	14	S	CCACTCCATgAggTATTTCTT	184	512-528	14	AS	CgCACgggCCgCCTCCA
174	241-259	14	S	CCggAgTATTgggACCTgC	207	294-311	14	AS	CCgAgAgAgCCTgCggAA
187	217-234	14	S	gCgCCgTggATAgAgCAA	208	293-311	14	AS	ACCgAgAgAACCTgCggAT
188	190-206	14	S	gCCgCgAgTCCgAggAC	212	538-556	14	AS	CCTCCAggTAggCTCTgTC
189	254-272	15	S	ACCggAACACACAgATCTg	213	387-402	14	AS	gAggAggCgCCCgTCg
192	254-272	14	S	ACCgggAgACACAgATCTC	214	419-435	14	AS	CTTgCCgTCgTAggCgg
193	243-261	14	S	ggAgTATTgggACCggAAC	215	420-438	14	AS	ATCCTTgCCgTCgTAggCT
194	268-285	14	S	AACATgAAggCCTCCgCg	216	435-454	14	AS	CgTTCAgggCgATgTAATCT
195	253-272	15	S	gACCggAACACACAgATCTT	217	544-561	14	AS	CgTgCCCTCCAggTAggT
196	292-309	14	S	TACCgAgAgAACCTgCgC	218	559-576	14	AS	gAgCCACTCCACgCACTC
197	230-246	14	S	AgCAggAggggCCggAA	219	572-589	14	AS	CCAggTATCTgCggAgCg
198	78-97	14	S	CCACTCCATgAggTATTTCg	220	603-619	14	AS	CCgCgCgCTCCAgCgTg
202	124-141	14	S	ggggAgCCCCgCTTCATT	221	605-622	14	AS	TACCAgCgCgCTCCAgCT
203	265-283	14	S	CAgATCTACAAggCCCAgg	223	353-372	14	AS	gCCATACATCCTCTggATgA
205	83-103	14	S	CCATgAggTATTTCTACACCg	224	361-379	14	AS	CgTCgCAgCCATACATCAC
206	253-272	15	S	gACCggAACACACAgATCTA	225	527-544	14	AS	CTCTCAgCTgCTCCgCCT
207	294-311	14	S	CCgAgAgAgCCTgCggAA	227	369-385	14	AS	gCCCCACgTCgCAgCCg
207	294-311	14	S	CCgAgAgAgCCTgCggAA	228	411-428	14	AS	TCgTAggCgTCCTggTgg
208	293-311	14	S	ACCgAgAgAACCTgCggAT	229	499-516	14	AS	CTCCAACTTgCgCTgggA
208	293-311	14	S	ACCgAgAgAACCTgCggAT	232	246-265	14	AS	gTgTgTTCCggTCCCAATAT
209	189-206	14	S	CgCCgCgAgTCCgAgAgA	234	319-337	14	AS	CgCTCTggTTgTAgTAgCg
239	406-423	14	S	gggTACCAgCAggACgCT	236	354-371	8	AS	CCATACATCgTCTgCCAA
240	259-278	14	S	gAgACACAgAAgTACAAgCg	237	302-318	14	AS	gCgCAggTTCCgCAggC
242	295-312	14	S	CgAgAgAgCCTgCggAAC	238	559-576	14	AS	gAgCCACTCCACgCACAg
243	192-209	14	S	CgCgAgTCCgAggATggC	241	463-479	14	AS	gCCgCggTCCAggAgCT
246	78-97	14	S	CCACTCCATgAggTATTTCC	244	571-588	14	AS	CAggTATCTgCggAgCCC
493	162-180	14	S	CACgCAgTTCgTgCggTTT	247	463-479	14	AS	gCggCggTCCAggAgCg
251	300-317	14	S	ggACCTgCggACCCTgCT	249	538-556	14	AS	CCTCCAggTAggCTCTCAA
271	125-142	14	S	gggAgCCCCgCTTCATCT	250	299-316	14	AS	gCAggTTCCgCAggCTCT
272	189-206	13	S	CgCCACgAgTCCgAggAA	276	499-515	14	AS	TCCCACTTgCgCTgggT
278	300-317	14	S	gAgCCTgCggACCCTgCT	277	387-403	14	AS	ggAggAAgCgCCCgTCg
280	149-167	14	S	gCTACgTggACgACACgCT	281	280-298	16	AS	CTCggTCAgTCTgTgCCTT
284	247-265	14	S	TATTgggACgAggAgACAg	282	280-299	14	AS	TCTCggTAAgTCTgTgCCTT
286	186-203	15	S	CgACgCCgCgAgCCAgAA	285	412-430	7	AS	CgTCgTAggCgTACTggTC
288	283-302	14	S	TCACAgACTgACCgAgCgAA	287	559-576	14	AS	gAgCCCgTCCACgCACTC
290	264-282	14	S	ACggAATgTgAAggCCCAg	298	423-443	14	AS	ATgTAATCCTTgCCgTCgTAA
291	184-200	17	S	AgCgACgCCgCgAgCCA	299	555-572	14	AS	CACTCCACgCACgTgCCA
292	239-257	14	S	ggCCggAgTATTgggACgA	300	448-466	14	AS	AgCgCAggTCCTCgTTCAA
294	283-302	14	S	TCACAgACTgACCgAgAgAg	301	414-431	14	AS	CCgTCgTAggCgTgCTgT
294	283-302	14	S	TCACAgACTgACCgAgAgAg	302	453-471	14	AS	CCAAgAgCgCAggTCCTCT
295	110-126	14	S	CCCggCCCggCAgTggA	303	527-544	14	AS	CTCTCTgCTgCTCCgCCg

Table 1b. Class I Primers (continued)

296	222-240	14	S	gTggATAgAgCAggAgggT	315	368-384	14	AS	CCCCAggTCgCAgCCAC
313	252-270	14	S	ggACCgggAgACACAgAAC	317	526-543	14	AS	TCTCAgCTgCTCCgCCgT
366	294-312	14	S	CCgAgTgAACCTgCggAAA	377	538-556	14	AS	CCTCCAggTAggCTCTCCA
367	322-341	14	S	TACTACAACCAgAgCgAggA	378	853-870	14	AS	CAgCCCCTCgTgCTgCAT
368	284-302	13	S	CACAgACTgACCgAgTgAg	379	601-618	14	AS	CgCgCgCTgCAgCgTCTT
369	196-213	14	S	AgTCCAAgAggggAgCCg	382	538-556	14	AS	CCTCCAggTAggCTCTCAg
371	78-98	14	S	CCACTCCATgAggTATTTCTC	388	361-379	14	AS	ggTCgCAgCCAAACATCCA
395	190-206	7	S	gCCgCgAgTTCgAgAgg	389	589-608	14	AS	AgCgTCTCCTTCCCATTCTT
402	275-292	14	S	AggCCCACTCACAgACTC	392	419-436	14	AS	CCTTgCCgTCgTAggCgA
433	344-363	14	S	ggTCTCACACCCTCCAgAAT	393	412-429	14	AS	gTCgTAggCgTCCTggTC
434	78-98	14	S	CCACTCCATgAggTATTTCAC	394	363-382	14	AS	CCACgTCgCAgCCATACATT
435	303-319	14	S	CCTgCgCACCgCgCTCC	399	583-601	14	AS	CCTTCCCgTTCTCCAggTg
451	406-423	14	S	gggTACCggCAggACgCT	400	418-435	14	AS	CTTgCCgTCgTAggCgTC
475	264-282	14	S	ACggAAAgTgAAggCCCAg	414	362-380	14	AS	ACgTCgCAgCCATACATCA
572	273-292	14	S	CAAggCCAAggCACAgACTT	425	412-430	7	AS	CgTCgTAggCgTACTggTT
573	297-314	14	S	AgAgAACCTgCggATCgC	429	259-278	14	AS	gCCTTCACATTCCgTgTgTT
574	284-301	14	S	CACAgACTgACCgAgAgg	431	559-575	14	AS	AgCCCgTCCACgCACCg
575	231-247	14	S	gCAggAggggCCggAgT	433	344-363	14	AS	ggTCTCACACCCTCCAgAAT
581	273-292	14	S	CAAgACCAACACACAgACTT	438	317-335	14	AS	CTCTggTTgTAgTAgCggA
625	239-257	14	S	ggCCggAgTATTgggACCA	486	257-276	14	AS	CTTCACATTCCgTgTCTCCT
751	113-127	14	S	ggCCCggCCgCgggg	494	299-316	14	AS	gCAgggTCCCCAggTCCA
753	434-453	14	S	AggATTACATCgCCCTgAAA	517	363-380	14	AS	ACgTCgCAgCCgTACATg
754	225-243	14	S	gATAgAgCAggAgAggCCT	530	311-329	14	AS	TTgTAgTAgCggAgCgCgA
824	397-414	14	S	TTCCTCCgCgggTACCAC	572	273-292	14	AS	CAAggCCAAggCACAgACTT
1168	725-742	14	S	AgCgggATggggAggACT	573	297-314	14	AS	AgAgAACCTgCggATCgC
1173	368-385	14	S	ATggCTgCgACgTggggT	574	284-301	14	AS	CACAgACTgACCgAgAgg
1191	555-571	14	S	gggCACgTgCgTggACg	575	231-247	14	AS	gCAggAggggCCggAgT
1605	342-361	14	S	CAggTCTCACACCCTCCAgT	752	282-300	14	AS	CACTCggTCAgTCTgTgAC
1719	Int 143-60	10	S	gCGAggggACCgCAggC	818	485-502	14	AS	gggTgATCTgAgCCgCCT
126	477-494	14	AS	TgAgCCgCCgTgTCCgCA	1167	916-933	14	AS	gATgCCCACgATggggAC
127	361-379	7	AS	ggTCgCAgCCATACATCCA	1171	Int 2-518	14	AS	gggTgTgAgAACCTggCCT
143	368-385	14	AS	gCCCCAggTCgCAgCCAA	1189	620-638	14	AS	TATgTgTCTTgggggggT
145	559-576	14	AS	gAgCCACTCCACgCACTC					
146	539-557	14	AS	CCCTCCAggTAggCTCTCT					
157	474-490	14	AS	CCgCCgTgTCCgCggCA					

Table 2a. Information for Primer Mixes 1-96

Lane	PM Number	Sense primer	Sense conc. †	antisensense primer	Antisense conc.	Amplicon size	Locus	Alleles amplified
1	151	286	5	431	5	629	A	*0101 *0102 *0103 *0104N
2	290	173	5	400	5	597	A	*0101 *0103 *0104N *03011 *0301 *0301 *0302 *0303 *0304 *3201 *3202 *3203 *3601 *7401 *7402 *7403 *8001
3	292	753	5	249	5	122	A	*02011 *0204 *0206 *0207 *0209 *0210 *0211 *0214 *0215 *0216 *0217 *0217 *0218 *0220 *0221 *0225 *0226 *0229 *0230 *0231 *0232 *2301 *2413 *2418 *6901
4	1048	402	10	249	10	521	A	*02011 *0204 *0206 *0207 *0209 *0210 *0214 *0215 *0216 *0217 *0217 *0218 *0220 *0221 *0224 *0225 *0226 *0229 *0232N
5	3	296	5	302	5	489	A	*02011 *0202 *0203 *0204 *0205 *0206 *0207 *0208 *0209 *0210 *0211 *0212 *0213 *0214 *0215 *0216 *0217 *0217 *0218 *0219 *0220 *0221 *0222 *0225 *0226
								*0227 *0229 *0230 *0231 *0232N
6	1021	1173	5	299	5	204	A	*03011 *03012 *03013 *0302 *0304 *2418
7	4	291	7	299	7	628	A	*03011 *03012 *03013 *0302 *0303 *0304 *2418
8	294	290	5	249	5	532	A	*03011 *03013 *0303N *0304 *2901 *2902 *2903 *2904 *3001 *3402 *6901
9	2	286	3	168	3	630	A	*3601
10	5	284	10	302	10	464	A	*2301 *240210 *240210 *2403 *2404 *2405 *2406 *2407 *2409 *2410 *2411 *2413 *2414 *2417 *2418 *2422
11	296	284	5	244	5	581	A	*2301 *240210 *240210 *2404 *2405 *2406 *2407 *2409 *2411 *2413 *2414 *2417 *2421
12	6	292	10	249	10	557	A	*2301 *2413 *2416 *2418 *3007
13	7	292	10	170	10	557	A	*240210 *240210 *2403 *2404 *2405 *2407 *2409 *2410 *2411 *2414 *2417 *2421
14	945	208	5	146	5	504	A	*240210 *240210 *2403 *2405 *2407 *2408 *2409 *2410 *2411 *2414 *2417 *2421
15	905	292	5	1171	5	519	A	*240210
16	958	1191	5	1189	5	671	A	*2411N
16	958	1191		1167	5	1087	A	*240210 *240210 *2404 *2405 *2406 *2407 *2408 *2409 *2411 *2413 *2414 *2417
16	958	1168	5	1167		329	A	*2409N
17	8	239	4	167	4	170	A	*2501 *2502 *2601 *2603 *2605 *2607 *2608 *2611 *2612 *4301 *6601
				145	4	170		*6602 *6603
18	9	193	10	298	10	440	A	*2501 *2502 *2601 *2602 *2603 *2604 *2605 *2606 *2608 *2609 *2611 *2612 *3401 *3402 *6601 *6602 *6603
19	10	294	10	298	10	400	A	*2501 *2502
20	11	288	10	298	10	400	A	*2601 *2602 *2604 *2607 *2608 *2609 *2611 *2612 *4301
21	12	174	10	298	10	442	A	*4301
22	13	239	10	168	10	170	A	*2609 *3401 *3402
22	13	451	10	168	10	170	A	*3103 *3104
23	14	290	10	298	10	419	A	*2502 *3402 *6601 *6602
23	14	475	10	298		419	A	*3401
24	225	290	5	303	5	520	A	*1101 *1102 *1104 *1105
24	225	290	5	167	5	552	A	*1101 *1102 *1103 *1105 *2502 *6601
25	295	751	10	752	10	187	A	*03011 *03012 *03013 *0302 *0303 *0304 *1101 *1103 *1104 *1105 *2407 *2502 *2901 *2902 *2903 *2904 *3401 *3402 *6601 *6602 *6801 *6801 *6802 *6806 *6807 *6808
								*6809 *6810 *6901
26	152	174	5	300	5	465	A	*2901 *2902 *2903 *2904
27	18	295	5	301	5	561	A	*3001 *3002 *3003 *3004 *3006 *3007

Table 2a. Information for Primer Mixes 1-96 (continued)

28	298	754	5	249	5	571	A	*3001	*3002	*3007	*31012	*3102 *3103 *3104
29	184	434	5	486	5	198	A	*31012	*3103	*3104		
30	347	173	5	414	5	542	A	*0103	*0204	*3201	*3202	*3203 *7401 *7402 *7403
31	1514	493	15	530	15	167	A	*3201	*3202	*3203		
32	154	434	10	429	10	200	A	*3301	*3303	*3304		
33	309	1	10	399	10	578	A	*3301	*3304			
34	188	493	10	494	10	154	A	*7401	*7402			
35	226	193	5	302	5	468	A	*68011	*68012	*6802	*68031	*6803 *6804 *6805 *6806 *6807 *6808 *6809 *6901
36	24	290	3	171	5	383	A	*6901				
37	25	367	10	287	10	494	A	*8001				
38	279	625	5	400	10	436	A	*0101	*0102	*0103	*0104N	*0301 *0301 *0301 *0302 *0303 *0304 *1101 *1102 *1103 *1104 *1105 *3101 *3102 *3201 *3202 *3203 *3601 *7401 *7402 *7403
38	279	292	5	400		436	A	*2416	*2417	*6810	*8001	
39	34	193	10	221	10	619	B	*07021	*07022	*07023	*0703	*0704 *0705 *0706 *0707 *0708 *0709 *0710 *0711 *0712 *4806 *8101
40	273	203	10	285	10	405	B	*07021	*07022	*07023	*0704	*0707 *0709 *0711 *0712 *5603
41	272	203	10	425	10	405	B	*0705	*0706	*4201	*4202	*5504 *5508 *5605 *8101
42	1185	271	10	517	10	495	B	*07021	*07022	*07023	*0703	*0704 *0705 *0706 *0708 *0709 *0710 *0711 *0713 *0801 *0802 *0803 *0804 *0805 *0806 *0807 *0808 *1405 *3903 *3914 *4201 *4202 *4801
									*4804	*4805	*4806	*8101
43	360	242	10	220	10	564	B	*0801	*0804	*0805	*0806	*0807 *0808 *4101 *4102 *4103 *4201 *4202
44	36	195	10	212	10	543	B	*0801	*0802	*0803	*0807	*0808 *4406 *5108
45	155	195	10	220	10	606	B	*0801	*0802	*0803	*0806	*0807 *0808N
46	37	280	10	225	10	635	B	*2704	*2706	*2710	*4005	*4015 *4016 *4901 *5001 *5002
47	38	208	10	215	10	385	B	*4901	*5115	*5901		
48	39	246	10	215	10	600	B	*4501	*4901	*5001	*5002	
49	40	207	5	219	5	535	B	*1514	*4409	*4501	*4502	*5002 *8201
50	304	192	10	212	10	542	B	*3701	*4101	*4102	*4103	*4402 *4405 *4408 *4409 *4411 *4501 *4502
51	160	202	10	285	10	546	B	*1804				
51	160	202		393	10	545	B	*4406				
51	160	272	10	285		481	B	*1546	*3519	*4003	*4009	*4018 *4020 *4405
51	160	272		393		480	B	*4402	*44031	*44032	*4404	*4407 *4409 *4411
52	231	196	5	212	5	504	B	*0802	*4402	*4405	*4408	*4411
53	228	196	5	400	5	383	B	*2701	*4402	*44031	*44032	*4404 *4407 *4408 *4411
54	229	196	5	382	5	504	B	*1301	*1302	*1303	*1304	*1536 *2701 *4403 *4403 *4407 *4410
55	288	196	5	227	5	333	B	*0802	*38021	*38022	*4402	*4403 *4404 *4405 *4408 *4411
56	42	192	4	220	5	605	B	*4101	*4102	*4103		
57	43	272	10	276	10	566	B	*1546	*3519	*4002	*4003	*4004 *4005 *4006 *4008 *4009 *4011 *4013 *4014 *4015 *4016 *4018 *4019 *4020 *4101 *4102 *4103 *4402 *4403 *4403 *4404 *4405 *4407
									*4409	*4410	*4411	*4501 *4502 *4701 *4702 *4703 *4901 *5001 *5002
58	44	192	4	247	10	465	B	*1533	*40011	*40012	*4002	*4003 *4004 *4005 *4006 *4009 *4010 *4011 *4012 *4014 *4015 *4016 *4018 *4019 *4020 *4101 *4102 *4103 *4801 *4803 *4804 *4805
59	45	272	10	218	10	627	B	*40011	*40012	*4002	*4003	*4004 *4006 *4007 *4008 *4009 *4010 *4011 *4013 *4014 *4016 *4018 *4019 *4020 *4701 *4702 *4703
60	46	280	10	229	10	607	B	*40011	*40012	*4007		
61	47	243	10	215	10	486	B	*1301	*1302	*1303		
62	48	197	10	127	10	389	B	*1401	*1402	*1403	*1404	
63	49	205	10	232	10	182	B	*1402	*1403	*1405	*3526	*3904
64	50	207	10	217	10	507	B	*1548	*39011	*39013	*39021	*3902 *3903 *3904 *3905 *3906 *3906 *3907 *3908 *3909 *3910 *3911 *3912 *3913 *3914 *3915 *6701 *67012

Table 2a. Information for Primer Mixes 1-96 (continued)

| | | |

Table 2b. Information for Primer Mixes 97-192

Lane	PM Number	Sense primer	Sense conc. †	antisensense primer	Antisense conc.	Amplicon size	Locus	Alleles amplified
97	80	188	10	237	10	128	B	*1522 *1801 *1802 *1803 *1804 *1805 *1807 *3501 *3502 *3503 *3504 *3505 *3506 *3507 *3508 *3509 *3509 *3510 *3511 *3512 *3513 *3514 *3515 *3516 *3517 *3518 *3520 *3521 *3522 *3523 *3524 *3528 *7801 *7802 *7802 *7803 *7804
98	81	195	10	213	10	389	B	*3501 *3503 *3504 *3505 *3506 *3507 *3508 *3509 *3509 *3511 *3514 *3515 *3517 *3518 *3519 *3521 *3523 *3524 *3525 *3527 *5301 *5302 *5303
98	81	195		277	10	390	B	*3502
99	305	195	5	226	5	416	B	*0708 *0807 *1508 *1522 *1529 *1807 *3501 *3503 *3505 *3507 *3508 *3511 *3514 *3515 *3517 *3519 *3521 *3523 *3524 *3525 *3527 *5301 *5302 *5303
100	307	195	5	392	5	423	B	*3503 *3506 *5113
101	82	207	10	216	10	400	B	*15012 *1509 *5605 *7801 *7802 *7802 *7803 *7804
102	83	208	10	216	10	401	B	*51011 *51012 *51021 *51022 *5103 *5104 *5105 *5106 *5107 *5108 *5109 *5111 *5112 *5114 *5116 *5201 *52012
103	1055	202	20	236	20	487	B	*51011 *51021 *5103 *5107 *5108 *5109 *5110 *5111 *5112 *5113 *5114 *5116 *5201 *5605 *7801 *7802 *7803
104	84	193	5	216	5	451	B	*1509 *51011 *51012 *51021 *5102 *5103 *5104 *5105 *5106 *5107 *5108 *5109 *5111 *5112 *5114 *5116 *5605 *7801 *7802 *7802 *7803 *7804
105	311	195	5	399	5	588	B	*1807 *3521 *51011 *51012 *5103 *5104 *5106 *5108 *5109 *5111 *5112 *5113 *5114 *5116 *5302 *7801 *7802 *78022
106	85	192	5	216	7	440	B	*15012 *52011 *52012
107	1112	1719	5	438	5	421	B	+/- Bw4: *0802-3,*1301-4,*1513/16/17/23/24/36/1543,*2701-7/9-14,*3701-2,*3801-3,*4013/19,*4402-8/10/11,*4701/3,*4901,*5101-16,, *52011,*52012,*5301-3,*5701-5,*5801-2,*5901
108	1113	1719	5	237	5	404	B	+/- Bw6: *07021,*07022,*07023,*0703,*0704,*0705,*0706,*0707,*0708,*0709,*0710,*0712,*0713,*0801-8N,*1401-5,*1501-12/14-15/18-22/ 25-35/37-40/44-50,*1801-5/7,*2708/12,*3501-26/28,*3901-15,*4001-16/20,*4101-3,*4201-2,*4409,*4501-2,*4601,*4702-3, *4801-6,*5001-2,*5401,*5501-5/7/8,*5601-5,*67011,*67012,*7301,*7801-4,*8101,*8201,
109	86	368	2	315	2	340	Cw	*0102 *0103
110	87	366	10	145	10	522	Cw	*02021 *02022 *02023 *02024 *0203 *1701 *1702
111	88	368	7	389	7	564	Cw	*0302 *03031 *03032 *03041 *0304 *0305 *0306 *0308 *0309
112	89	366	7	143	7	331	Cw	*04011 *04012 *0402 *0403 *0404 *0405 *0406 *1801 *1802
113	275	271	10	143	10	500	Cw, B	B*8201 *0708 *1801 *1802
114	90	366	10	379	10	564	Cw	*0501
115	1016	240	10	818	10	483	Cw	*0501 *0502 *0802 *0804 *0805
116	91	367	10	127	10	297	Cw	*0602 *0603 *0604
117	1075	4	10	184	10	484	Cw	*0701 *0702 *0704 *0705 *0706 *0708 *0710 *0711
118	93	313	3	184	3	516	Cw	*0701 *0706 *0707
119	94	367	5	183	5	302	Cw	*0702 *0703 *0710
120	96	367	5	379	5	536	Cw	*0704 *0711 *0712
121	182	165	10	166	5	160	Cw	*0801 *0802 *0803 *0804 *0805
121	182	165		317	5	625	Cw	*0801 *0803
122	99	159	5	389	5	523	Cw	*03031 *03032

Table 2b. Information for Primer Mixes 97-192 (continued)

123	201	130	5	389	10	206	Cw	*0302
123	201	160	5	389		522	Cw	*0302 *03041 *03042 *0305 *0306 *0308 *0309
124	102	369	15	126	15	538	Cw	*12021 *12022
125	103	368	15	157	15	446	Cw	*1203 *1206
126	104	371	15	388	15	541	Cw	*14021 *14022 *1403
127	106	366	10	223	10	318	Cw	*1502 *1503 *1504 *15051 *1505 *1506
128	107	366	10	382	10	502	Cw	*0203 *0404 *0406 *0604 *0707 *1502 *1503 *1504 *1505 *1505 *1506 *1701 *1702
129	109	368	10	146	10	513	Cw	*1601
130	110	366	10	146	10	503	Cw	*1602
131	1017	368	10	127	10	335	Cw	*0102 *0103 *1203 *1206 *1402 *1402 *1403 *1601 *16041
132	111	366	10	377	10	502	Cw	*02021 *02022 *02023 *02024 *0602 *0603 *1204 *1204 *1205
133	1018	366	10	157	10	436	Cw	*12042 *1205
134	113	36	10	39	10	194	DRB1	*0101 *01021 *01022 *0104 *0105 *0106
135	197	36	10	38	10	254	DRB1	*01021 *01022 *0104 *0106
136	114	36	10	40	10	195	DRB1	*0103
137	115	41	10	252	10	206	DRB1	*15011 *15012 *15021 *15022 *1502 *1503 *1504 *1505 *1506 *1507 *1508
138	198	41	10	38	10	256	DRB1	*15011 *15012 *1503 *1504 *1505 *1506 *1507
139	282	41	10	42	10	196	DRB1	*15011 *15012 *15021 *15022 *1502 *1503 *1506 *1507 *1508
140	283	650	10	252	10	155	DRB1	*1503
141	284	41	10	651	10	196	DRB1	*1504
142	185	41	10	491	10	136	DRB1	*1507 *16011 *16012 *16021 *1602 *1603 *1604 *1605 *1607 *1608
143	162	68	10	255	10	211	DRB1	*03011 *03012 *03021 *03022 *0303 *0304 *0305 *0306 *0307 *0308 *0309 *0310 *0311 *0312 *0313 *0314 *1107
144	118	46	10	38	10	216	DRB1	*03011 *03012 *0304 *0306 *0308 *0310 *0311 *0312 *0313 *1327
145	119	44	10	37	10	188	DRB1/3	*03021 *03022 *0305 *0314 *1109 *1120 *1302 *1305 *1326 *1329 *1331 *1334 *1402 *1403 *1409 *1413 *1419 *1424 *1427 *1430 *1608 DRB3*0206
146	120	47	10	37	10	259	DRB1	*04011 *04012 *04051 *04052 *0407 *0408 *0409 *0414 *0416 *0417 *0419 *0420 *0421 *0424 *0426 *0428 *0429 *0430 *0431 *1122
146	120	47		38	10	259	DRB1	*0402 *04031 *04032 *0404 *0406 *0410 *0411 *0412 *0413 *0415 *0418 *0422 *0425 *0427 *0432 *1410
147	312	47	10	498	10	211	DRB1	*04011 *04012 *0402 *0409 *0413 *0414 *0416 *0421 *0426
148	1522	47	10	2510	10	173	DRB1	*04011 *04012 *0402 *04031 *0404 *0406 *0407 *0408 *0413 *0414 *0418 *0419 *0420 *0421 *0422 *0423 *0425 *0427 *0431 *0432
149	314	47	10	500	10	221	DRB1	*04031 *04032 *0406 *0407 *0411 *0417 *0420 *0427
150	315	47	10	501	10	222	DRB1	*04011 *04012 *0402 *0404 *0405 *0405 *0408 *0409 *0410 *0413 *0414 *0415 *0416 *0419 *0421 *0423 *0424 *0426 *0428 *0429 *0430 *0432 *1122
151	1396	48	10	258	10	203	DRB1	*0701 *0703 *0704
152	122	50	10	51	10	213	DRB1	*0801 *08021 *08022 *08032 *0804 *0804 *0804 *0806 *0807 *0808 *0809 *0810 *0811 *0812 *0813 *0815 *0816 *0817 *0819 *1415
152	122	50		102	10	161	DRB1	*0801 *08032 *0805 *0806 *0810 *0812 *0816 *0817 *0818
153	320	50	10	491	10	130	DRB1	*0801 *08021 *08022 *08032 *0804 *0804 *0804 *0805 *0806 *0807 *0808 *0809 *0810 *0811 *0812 *0813 *0815 *0816 *0818 *0819 *1404 *1411 *1415 *1428 *1431
154	1051	1634		1635		136	DRB1	*08032 *0810 *0812 *0814 *0815 *0818 *0819 *1118 *1119 *1131 *1312 *1313 *1330
155	133	263		58		165	DRB1	*1117 *1401 *1404 *1405 *1407 *1408 *1410 *1411 *1414 *1423 *1426 *1428 *1433
155	133	263		51		164	DRB1	*0809 *0821 *1318 *1415
156	227	46		49		192	DRB1	*09012
157	124	53	10	37	10	203	DRB1	*1001
158	125	68	10	54	10	175	DRB1	*0308 *11011 *11012 *11013 *1102 *1103 *1104 *1104 *1106 *1107 *1108 *1108 *1109 *1110 *1111 *1112 *1113 *1114 *1115 *1116 *1117 *1118 *1119 *1120 *1121
158	125	273	10	54		173	DRB1	*1123 *1124 *1125 *1126 *1127 *1128 *1129 *1132 *1134 *1105 *1204 *1411
159	1052	1630	10	37	10	118	DRB1	*11011 *11012 *11013 *1105 *1108 *1108 *1109 *1110 *1111 *1112 *1114 *1115 *1119 *1120 *1122 *1123 *1124 *1126 *1127 *1128 *1129 *1130 *1131 *1132 *1133
160	202	44		54		104	DRB1	*0308 *1109 *1116 *1120
161	126	50	15	256	15	162	DRB1	*1201 *12021 *12022 *12032 *1205 *1206

Table 2b. Information for Primer Mixes 97-192 (continued)

162	280	50		55		247	DRB1	*0812	*1201	*12021	*12022	*1204 *1205 *1206 *1428
163	183	68		259		210	DRB1	*1102	*1103	*1111	*1114	*1116 *1120 *1121 *1301 *1302 *1304 *1308 *1315 *1316 *1319 *1320 *1322 *1323 *1324 *1327 *1328 *1329 *1331 *1332 *1334 *1416
163	183	273		259		208	DRB1	*1317				
164	127	263	10	259	10	152	DRB1	*1116	*1120	*1301	*1302	*1308 *1316 *1320 *1327 *1328 *1329 *1331 *1332 *1334 *1416
164	127	263		252	10	151	DRB1	*1309				
165	168	44	10	40	10	129	DRB1	*1116	*1120	*1301	*1302	*1306 *1310 *1315 *1316 *1327 *1328 *1331 *1332 *1334
165	168	44		485	10	139	DRB1	*1310	*1419	*1421		
166	169	44	10	258	10	136	DRB1	*1109	*1305	*1306	*1318	*1326 *1403 *1412 *1427 *1608
166	169	44		485	10	139	DRB1	*1310	*1419	*1421		
167	167	68	10	485	15	210	DRB1	*13031	*13032	*1310	*1333	*1419 *1421
168	170	68	10	102	15	170	DRB1	*0312	*13031	*13032	*1304	*1312 *1313 *1321 *1330 *1332 *1333 *1413
169	131	68	10	104	15	200	DRB1	*0820	*11011	*11012	*11013	*1103 *1104 *1104 *1106 *1109 *1110 *1111 *1112 *1115 *1123 *1124 *1125 *1127 *1128 *1129 *1132 *1133 *1135 *1305 *1307 *13072
170	1525	68	10	2510	10	173	DRB1	*03011	*1311	*1314	*1318	*1321 *1324 *1326 *1422 *1425 *1427
									*03012	*03021	*03022	*0303 *0304 *0305 *0306 *0307 *0309 *0311 *0314 *0820 *1301 *1302 *1305 *1306 *1307 *1308 *1309 *1310 *1311 *1314 *1315 *1316
									*1318	*1319	*1320	*1322 *1323 *1324 *1325 *1327 *1328 *1329 *1334 *1402 *1403 *1406 *1409 *1412 *1414 *1417 *1419 *1420 *1421 *1423 *1424 *1427
									*1429	*1430	*1433	
171	1138	68	15	58	15	223	DRB1	*1117	*1401	*1405	*1407	*1408 *1414 *1418 *1423 *1426 *1433
172	1151	1631	15	38	15	122	DRB1	*0310	*1401	*1404	*1410	*1416 *1426 *1428 *1431 *1432
173	134	68	15	107	15	171	DRB1	*0310	*1401	*1407	*1416	*1422 *1425 *1426 *1432
174	1526	2489	10	51	10	152	DRB1	*1318	*1403	*1412	*1427	
174	1526	2489		39	10	129	DRB1/3	*03011	*03012	*03021	*03022	*0303 *0305 *0306 *0307 *0308 *0310 *0311 *0312 *0313 *0314 *1402 *1406 *1409 *1413 *1417 *1418 *1419 *1421 *1429 *1430
									*1433	DRB3*0206		
175	186	61	10	492	10	172	DRB5	*01011	*01012	*0102	*0103	*0104 *0105 *0106 *0107 *0108 *0109 *0202 *0203 *0204
176	164	69	10	268	10	221	DRB3	*01011	*01012	*01013	*01014	*0103 *0104 *0105
177	1079	1674	10	255	10	214	DRB3	*0102				
178	138	76	10	151	10	116	DRB3	*0201	*0202	*0203	*0205	*0206 *0207 *0208
179	1082	70	10	256	10	171	DRB3	*0301	*0302	*0303		
180	1049	60	10	38	10	212	DRB4	*01011	*0102	*0103	*0104	*0105
181	140	283	5	314	5	143	DRB4	*010110	*0102	*010310	*0104	*0105 *0201N
182	1054	270	5	866	5	146	DRB4	*0103102N				
183	142	77	10	350	10	194	DQB1	*0305				
184	143	82	10	112	10	197	DQB1	*0201	*0202	*0203		
185	144	77	20	78	20	207	DQB1	*0401	*0402			
186	145	79	5	152	5	206	DQB1	*0501	*0502	*05031	*05032	*0504
187	146	347	10	111	10	248	DQB1	*06011	*06012	*06013		
187	146	348	10	111		139	DQB1	*0602	*0603	*0610	*06111	*0611 *0613 *0614
188	147	349	10	350	10	175	DQB1	*0603	*06041	*06042	*06051	*0605 *0607 *0608 *0609 *0611 *0612 *0614
188	147	349		351	10	175	DQB1	*0606				
189	148	181	20	112	20	207	DQB1	*03011	*03012	*0304	*0309	
190	318	88	10	350	10	178	DQB1	*0302	*03032	*03033	*0307	*0308 *0602 *0603 *0604 *0604 *0605 *0605 *0607 *0608 *0609 *0610 *0611 *0611 *0612 *0613 *0614 *0615
191	362	353	10	831	10	121	DQB1	*0302	*0307	*0308		
192	268	1465	2	1466	2	130	DQB1	*0203	*03032	*03033	*0306	

†Primer volume in microlitres per ml of using 2000ug/ml stocks (see text on primer testing)

PCR-SSOP, Class I and Class II (DRB1)

Derek Middleton

Principle/Purpose

Described in this chapter is the sequence specific oligonucleotide probe (SSOP) method. This method is also directly applicable to defining other polymorphic loci within the MHC or elsewhere in the genome. The basis of this method is the specific amplification of the HLA-locus by polymerase chain reaction and the subsequent probing of this product by sequence specific oligonucleotide probes. Most of the vast polymorphism of the HLA system results from conversion events whereby small nucleotide sections of one allele (usually no more that 100 bases long) are transferred to another allele. Thus, many of the sequences tend to be shared by alleles and are not allele specific, necessitating the use of probes which are sequence specific. In order to differentiate the alleles, a battery of probes is required and it is the pattern of reactivity of these probes which distinguishes the HLA alleles.

The detection system used in this laboratory consists of labelling the probes with digoxigenin (DIG) and detecting the hybridization of these probes to a complementary sequence present in the PCR amplified HLA allele of an individual by adding an anti-digoxigenin antibody conjugated with alkaline phosphatase (ALP). The ALP then uses disodium 3-(4-methoxyspiro[1,2-dioxetane-3,2′-(5′-chloro)tricyclo[3.3.1.1]decan}-4-yL) phenyl phosphate (CSPD) as its chemiluminescent substrate and the light emitted is detected by autoradiography.

To define all alleles at any specific locus at the same time would require a large number of probes. Although each allele group has a specific probe pattern, the combined probe pattern of two alleles present in a heterozygous individual can be identical to the combined probe pattern of another heterozygous individual with two different alleles. In addition, the system would constantly need to be updated to take account of newly discovered alleles. In this laboratory we use a two-tier SSOP system. The first level of resolution is equivalent to very good serology, whereby the allele group is defined, e.g., HLA-A*02. Thereafter, depending on the initial type, a second PCR specific for a group of alleles is performed and a further set of probes used to give definition to the allele level. This keeps the required number of probes to a minimum and, except for exceptional circumstances, only the high resolution system needs alteration to take account of new alleles.

In this chapter details of primers, master mixes, amplification conditions and probes are only given for the first medium level resolution systems for HLA-A, -B, -C, and -DR. Anyone interested in details of our second high resolution allele level systems should contact *derek.middleton@bll.n-i.nhs.uk* for details.

The method of SSOP is described in eight sections. Instruments and reagents are listed only at first time of use.

Specimens

In this laboratory genomic DNA is isolated by the salting-out method, but other methods may be used. Blood is preferentially collected in Na_2 EDTA. Heparin is avoided. We do not routinely determine the concentration of DNA in each isolation. When isolating DNA the amount of TE buffer added to the pellet of DNA is judged by eye. However, we assess approximately 10% of samples to ensure that the DNA is at an appropriate concentration. For our methods we normally have the DNA concentration at approximately 0.2 µg/µl.

Protocols

A. PCR AMPLIFICATION

Purpose

To amplify a defined region of individual HLA loci suitable for differentiating HLA alleles. The primers for HLA-A, -B and -C loci give a locus-specific product covering exons 2 and 3 and the primer for HLA-DR gives a product from exon 2 (Table 1). This product is not specific for the HLA-DRB1 locus and amplifies alleles of other HLA-DR loci (e.g., HLA-DRB3 locus), making it necessary to include a further amplification for alleles of HLA-DRB1*03, -DRB1*11, -DRB1*13 and -DRB1*14. This is referred to as the HLA-DRB3/11/6 group. The reason for two 3′ end primers for HLA-B is because HLA-B*7301 differs in intron 3 from all other known alleles at this locus and the extra primer is required to amplify this allele. In testing for the HLA-B*27 alleles only the extra primer is not required. The probe BL12 detects a sequence which is only found in HLA-B*27 alleles and HLA-B*7301. Thus, leaving out primer 3 BIN3-AC means that HLA-B*7301 is not amplified, and, consequently, the BL12 probe is specific for alleles of HLA-B*27.

Reagents

1. Cresol Red: 10 mg/ml, sodium salt, (Sigma, St Louis, Mo, C9877). Add 200 mg to some dH_2O taken from measured 20 ml dH_2O in a sterile tube. Resuspend in remaining volume. Filter sterilize and dispense into 1 ml aliquots and freeze at -20°C. Cresol red is included in PCR master mix to save time in adding at dot blotting stage.
2. dH_2O. Double distilled H_2O or equivalent. Note that dH_2O used to set up PCR is of ultra high purity quality.
3. $MgCl_2$. Supplied with *Taq* enzyme.
4. NH_4 Buffer. Supplied with *Taq* enzyme.
5. dNTPs. (Pharmacia Biotech, St Albans, England, 27-2094).
6. *Taq* enzyme (Bioline, London, England, M958013).

Supplies

1. Benchkote (Whatman 2300916)
2. Nunc tubes (5ml)
3. PCR Plates 96 well (Advanced Biotechnologies, Epsom, England, AB-0366).
4. Sodium hypochlorite (bleach) containing 2% chlorine.

Instrumentation

1. Gilson pipettes
2. Microcentrifuge
3. Perkin Elmer 9600 PCR Machine (but conditions can be determined for other machines).
4. Vortex mixer

Procedure

1. When setting up a PCR wear a separate lab coat, wear gloves and change them frequently and perform all work in pre-PCR room using dedicated equipment. Pipettes should not be removed from pre-PCR room. Pipettes are labeled according to reagents and must only be used for these reagents. The use of tips with filters is advisable.
2. When preparing the mastermix thaw out following reagents ($MgCl_2$, dNTPs, PCR buffer and appropriate primers). Vortex each reagent briefly and centrifuge in a microcentrifuge for 5 sec and place in an ice bucket (PCR buffer and $MgCl_2$ should be centrifuged for 2 min). *Taq* polymerase should always be added last, after vortexing and centrifuging, and just prior to dispensing the master mix. The aliquoted master mixes should not be left on the bench (maximum 15 min – *Taq* loses activity once diluted in buffer).
3. Switch on PCR machine for at least 10 min prior to use to allow the machine to heat up. PCR machine should be situated in post-PCR room.
4. Heat DNA samples to be tested to 60°C for 5-10 min, vortex and centrifuge for 5 sec in microcentrifuge.
5. Prepare 10 ml of mastermix for appropriate locus (Table 2). Use dH_2O of ultra high purity quality. Dispense 100 µl slowly into tubes of the 96 well plate. Take care to avoid splashes and air bubbles at the bottom of the tubes. When all tubes have been filled, cover the 96 well plate with a sterile microtiter tray lid.
6. Add 1µl DNA sample to each well from position 1A→1H, 2A→2H etc. Only one row at a time should be uncovered by the lid. Leave two wells with mastermix only, to act as negative controls and leave appropriate number of wells for control DNA. When a complete row of DNA samples have been added, place a strip of 8 caps over these samples and press down gently. When DNA samples have been added to all tubes and caps are in place, use a cap sealing tool to ensure that all caps are pushed firmly into place. Enough controls should be included so that each probe will have two positive reactions. In addition, control DNA should be included as negative controls. These contain alleles with sequences which are closely related to the sequence which the probe detects and with which the probe might cross-hybridize. This is especially important when initially determining the optimum conditions for the probe to work. To maintain consistency between membranes we try to use the same controls. If a laboratory finds it difficult to have a large enough supply of the same control DNA it may consider cloning control DNA by long range amplification (Curran et al. 1996). This gives material to use in as many tests as needed.
7. Centrifuge the plate for 1 min at 500 g, place in PCR machine and run appropriate cycle program (Table 3). After amplification, if the PCR samples are not to be processed immediately, store them at -20°C.
8. After setting up a PCR, wash work areas with sodium hypochlorite (bleach). Soak all racks used to hold samples in sodium hypochlorite for approx 30 min, and rinse thoroughly in water. Pipettes should be wiped with sodium hypochlorite, followed by dH_2O. Wipe microcentrifuge, vortex, freezer handle etc with sodium hypochlorite. Expose the working area, including pipettes etc, to UV light for 60 min.

B. Agarose Gel Electrophoresis of Amplified DNA

Purpose

To ensure that a suitable amplified product has been obtained.

Reagents

1. Agarose (Promega, V312A)
2. 0.5M EDTA, pH 8.0: Add 186.1 g of EDTA $Na_2 2H_2O$ in parts to 800 ml dH2O. Adjust the pH to 8.0 using 4M NaOH. Make up to 1 liter with dH_2O and sterilize by autoclaving.

3. Ethidium bromide (10 mg/ml, Sigma, E-1510).
4. Gel loading buffer (GLB): Add 8 g sucrose (slowly) to 10 ml dH_2O and mix by inversion until dissolved. Then add 1 ml 1M Tris (pH 7.6), 2 ml 0.5M EDTA, 1 ml 10% SDS, 0.02 g cresol red. Make up to 20 ml with dH_2O. Do not autoclave.
5. Size Marker Φx174/*Hae III* (0.1mg/ml, Promega, Southampton, England, G1761). Add 450 µl dH_2O to vial of the size marker. Add 20 µl of GLB to 12 µl (1.2 µg) of the size marker and 8 µl of TE buffer. Store at 40°C.
6. 1M Tris, pH 7.6: Add 242.28 g Tris base to 1400 ml dH_2O. Adjust the pH to 7.6 by adding 100 ml concentrated HCl (CAUTION – wear a mask and goggles and where possible perform this in a fume hood). Allow the solution to cool to room temperature before making final adjustments to the pH. Make up to 2 liters with dH_2O and sterilize by autoclaving. If the 1M solution has a yellow color, discard it and obtain better quality Tris. More than 100 ml concentrated HCl may be required.
7. Tris borate EDTA (10x) (TBE): Add 216 g Tris, 110 g Orthoboric Acid and 80 ml 0.5M EDTA to 1400 ml dH_2O. Adjust volume to 2 liters with dH_2O and sterilize by autoclaving.
8. Tris-EDTA (TE) Buffer (10mM Tris, 1mM EDTA pH 7.6): Combine 10 ml 1M Tris pH 7.6 with 2 ml 0.5M EDTA and make up to 1 liter with dH_2O. Sterilize by autoclaving and aliquot.

Supplies

1. Microcentrifuge tubes (1.5 ml, Elkay 000-MICR-150)
2. Saran Wrap (Genetic Research Instrumentation, Felsted, England, SW1).
3. Thermofast Plate (Advanced Biotechnologies, AB-0600).

Instrumentation

1. Camera fitted with Kodak Wratten 23A filter.
2. Combs (GIBCO BRL 1.0 mm thick 14 tooth 580-1081EF)
3. Gel Sealer and Casting Tray (Merck, 306/7252/12)
4. Octapipette (multichannel pipettor)
5. UV Transilluminator

Procedure

1. Add 4.5 g of agarose to 300 ml 1xTris-borate EDTA (TBE), boil and allow solution to cool to 65°C.
2. While agarose is cooling prepare 96-well-gel template by placing casting tray in the gel sealer – take care to ensure gel sealer is not over tightened otherwise casting tray may separate when agarose is added.
3. Place sealed casting tray on top of a levelling table and adjust the feet of the levelling table until the bubble in the "spirit level" is centred.
4. Once agarose has cooled to 65°C add 15 µl of ethidium bromide (10 mg/ml) and mix gently. (Ethidium bromide is mutagenic)
5. Pour the molten agarose solution into the level casting tray. Immediately push any air bubbles to edges of the template using a pipette tip.
6. Insert four 24 slot combs into the gel, with equal spacing between combs. Allow gel to set for approximately 1 hour at room temperature.
7. Add 1000 ml of 1x TBE to an electrophoresis tank. Carefully remove combs from gel. Remove gel from the gel sealer. Place gel in tank containing 1x TBE buffer. Ensure gel is covered by buffer to a depth of 2-3 mm.
8. Add 4 µl of each PCR product to a 96 well Thermofast plate. Ensure product is in each well. Add 8 µl GLB to each well. Spin plate for 1 min to ensure mixing.
9. Load 10 µl of size marker into first well of each of the four rows.
10. Using an octapipette carefully load 10 µl of sample into each well of the gel. Care must be taken to ensure the octapipette is orientated properly when adding the samples to the gel.
11. Place the lid of the electrophoresis system on to the electrophoresis tank, connect the electrodes to the power pack and electrophorese the samples at 250 V, 250 mAmp for 20 min.
12. Once electrophoresis is complete, remove the gel from the tank and photograph under UV light. Check size of PCR product against size marker to ensure correct product has been amplified (Table 1).

C. Dot Blotting

Purpose

To immobilize amplified product to a membrane prior to hybridization with probes. One membrane is made for each probe. In addition, extra membranes are useful to repeat hybridization if required. This laboratory now uses an automatic dot blot but has previously prepared membranes using a manifold method similar to that described elsewhere (Baxter-Lowe, 1993). Other laboratories may use other equipment or dot blot by hand.

Reagents

1. 0.4M NaOH
2. Saline sodium phosphate EDTA (20x) (SSPE): 3M NaCl, 0.2M NaH_2PO_4, 0.02M EDTA pH 7.4. Add 350.6 g NaCl followed by 48 g NaH_2PO_4 to approx 1600 ml dH_2O. Then add 80 ml 0.5M EDTA (pH 8.0). Adjust the pH to 7.4 using 4M NaOH. Adjust volume to 2 liters and sterlize by autoclaving.

Supplies

1. Nylon Membrane (Boehringer, 1417 240).
2. Whatman paper (3mm chr, 3030917)

Instrumentation

1. Robbins Hydra dot blotter (Robbins Scientific, 1029-60-1)

Procedure

1. After PCR product has been dispensed onto the membranes, allow to air dry for at least 20 min.
2. Carefully place membranes DNA side up onto 2 sheets thick (3MM) Whatman paper soaked in 0.4M NaOH. Leave for 10 min. When placing membranes onto Whatman paper – take care to ensure that: 1) membrane is not dragged over denaturation pad, 2) all of the membrane soaks up the 0.4M NaOH, and 3) there are no air bubbles beneath the membrane.
3. Transfer each membrane onto Whatman paper (3MM) soaked in 10x SSPE. Leave for 5 min.
4. Gently wash in 2x SSPE and allow to air dry for at least 25 min.
5. Wrap membranes in Saran Wrap and place (DNA face down) on UV transilluminator for 4 min. Ensure that all the UV lights are fully on during the procedure; do not switch transilluminator off between each step. Place a glass plate on top of membranes to hold them flat during this procedure. Store membranes wrapped in tin foil at +4°C if not using immediately.

D. Labeling of Oligonucleotides

Purpose

To label the 3′-end of the probes prior to hybridization using digoxigenin (DIG)-ddUTP. However, many of the probes used in this laboratory are DIG-labelled during their manufacture, adding the digoxigenin moiety to 5′ amino oligonucleotides by incubating with a digoxigenin ester under mild alkali conditions.

Reagents

The labeling reagents are obtained in a kit from Boehringer (Cat No: 1362372).

Procedure

1. Remove all reagents from freezer (except Terminal Transferase – this should be removed just before use) and allow to thaw. Vortex reagents briefly, and centrifuge in microcentrifuge for 5 sec.
2. Combine the following: 4 µl Reaction Buffer (5x), 4 µl $CoCl_2$ (25mM), 1 µl digoxigenin (DIG) -ddUTP (1mM), 1 µl Terminal Transferase (50 units), 100 pmoles probe. Make up to 20 µl with dH_2O. Vortex samples briefly, centrifuge in microcentrifuge for 5 sec, and incubate at 37°C for 30 min in a water bath.
3. Centrifuge for 5 sec in microcentrifuge and place on ice for 5 min. Add 80 µl dH_2O, vortex briefly, and centrifuge in microcentrifuge for 5 sec. Aliquot in volumes related to the amount of probe used (Tables 4-8) and store at -20°C.

E. Hybridization and SSPE Stringency Washes

Purpose

To enable the probes to hybridize to amplified DNA. In this laboratory we do not use tetramethylammonium chloride (TMAC) owing to its toxic properties and the fact that in our experience it does not necessarily mean the use of one wash temperature. In this laboratory one individual normally performs 16 hybridizations at the same time. Thus if a laboratory is defining alleles at four loci (HLA-A, -B, -C, -DR), probes can be selected for use at the same time according to their wash temperature. This eliminates the requirement for a large number of water-baths.

Reagents

1. Buffer 1 (4x): 0.4M maleic acid, 0.6M NaCl pH 7.5. Add 300 ml 4M NaCl and 400 ml 2M maleic acid followed by 200 ml 4M NaOH to approximately 800 ml dH_2O. Add 27 g NaOH pellets. (Note: A white precipitate forms when all reagents are added – this will disappear as the pH approaches 7). Cool to room temperature and adjust pH to 7.5 by adding 4M NaOH by drops. Adjust volume to 2 liters with dH_2O and sterilize by autoclaving.
2. Blocking Reagent: (Boehringer, Lewes, England, 1096176), 5% in Buffer 1. Prepare 2 liters of 1x Buffer 1 by combining 500 ml 4x Buffer 1 with 1500 ml dH_2O. Add 100 g Blocking Reagent in parts, with vigorous mixing using a magnetic stirrer, to approximately 1600 ml 1x Buffer 1. As the Blocking Reagent is supplied in 50 g containers, there is no need to weigh it out. Heat to 65°C until Blocking Reagent is dissolved, allow to cool to room temperature, and make up to 2 liters with Buffer 1. Sterlize by autoclaving, and store at 4°C.
3. Denhardt's Solution (50x): 1% polyvinyl pyrrolidone (PVP), 1% ficoll, 1% bovine serum albumin (BSA). Prepare 200 ml of 2% PVP and 2% ficoll by adding 4 g of each to 180 ml dH_2O. (CAUTION: PVP is harmful if inhaled. Prepare this solution in a fume hood.). Dissolve with gentle mixing and make up to 200 ml with dH_2O. Sterilize by autoclaving and cool to room temperature. Add 4 g of BSA to 200 ml of above solution slowly with gentle mixing. When the BSA has dissolved make up to 400 ml with dH_2O, mixing well. Filter solution through 0.45um filter, aliquot, and store at -20°C. (Note: Do not autoclave.) Leave to thaw at +4°C the evening before it is to be used.

4. N-Laurolysarcosine (1%). (CAUTION: Personal protective equipment should be worn when weighing N-laurylsarcosine.) Dissolve 10 g N-laurlysarcosine in approximately 800 ml dH_2O. Adjust volume to 1 liter with dH_2O and autoclave.
5. Sodium dodecyl sulfate (10%) (SDS). (CAUTION: This reagent is extremely harmful if inhaled. Wear personal protective equipment when working with SDS powder. Wash skin thoroughly if in contact with SDS. Wipe down work area after use. Preferably add SDS to dH_2O in fume hood.) Add 100 g of SDS in small amounts to approx 800 ml dH_2O. As SDS is supplied in 100g containers, there is no need to measure it. Apply heat (up to 68°C) if necessary to assist dissolution. Allow to cool to room temperature and adjust the volume to 1 liter. (Note: Do not autoclave.)
6. 2x SSPE/0.1% SDS: Combine 240 ml 20x SSPE and 24 ml 10% SDS. Make up to 2400 ml with dH_2O.
7. 5x SSPE/0.1% SDS: Combine 600 ml 20x SSPE and 24 ml 10% SDS. Make up to 2400 ml with dH_2O.
8. Hybridization Buffer: 192 ml 2% Blocking Reagent, 144 ml 6x SSPE, 48 ml 5x Denhardt's Solution, 48 ml 0.1% N-laurylsarcosine, 0.96 ml 0.02% SDS and make up to 480 ml with dH_2O.

Instrumentation

1. Robbins Gemini water-bath (Robbins Scientific, 1051-20-2).
2. Robbins hybridization incubator (Robbins Scientific, 1040-60-2).

Procedure

Each probe is simultaneously hybridized to two different membranes, each containing 96 DNA samples.

1. Roll membranes by hand lengthwise to form a cylinder. Place two membranes in a hybridization bottle. One membrane should have the DNA side of the membrane facing the glass, while the second membrane should have the DNA side facing inwards in the bottle.
2. Add 20 ml of freshly prepared Hybridization Buffer. Screw cap on tightly and clamp to the rotisserie of a Robbins incubator (pre-set at 45°C). Rotate the bottles for 1 hour.
3. Just before the incubation is complete, thaw appropriate aliquots of DIG-labeled oligonucleotide probe, vortex briefly and centrifuge for 5 sec in a microcentrifuge.
4. Add appropriate number of picomoles of probe (Tables 4-8) to 20 ml of pre-warmed (45°C) Hybridization Buffer and mix by inversion.
5. Remove the hybridization bottle from the incubator and pour off the Hybridization Buffer into a disposable collection container. Add 20 ml of Hybridization Buffer containing DIG-labelled probe and incubate bottle for 1 hour at 45°C.
6. Remove the bottle from the incubator and pour off the fluid into a disposable collection container.
7. Add 100 ml of 2x SSPE/0.1% SDS. Re-cap the bottle and place inside a Robbins incubator (pre-set to 25°C) and incubate for 10 min. Make sure temperature does not rise above this.
8. Discard the fluid and repeat Step 7.
9. Remove the bottle from the incubator. Uncap the bottle and, using forceps, carefully remove the membranes from the bottle, prior to discarding fluid, directly into a small plastic tray containing 200 ml 5x SSPE/0.1% SDS, which has been heated to the appropriate temperature (Tables 4-8). Place one membrane DNA side down and the other membrane DNA side up into the washing solution. Incubate with shaking for 40 min. Check temperature reading and record any variation on the hybridization record sheet. If the temperature varies more than 2°C above or below the required temperature, abandon this hybridization.
10. Remove the membranes from the tray, blot dry, but do not allow the membrane to dry out. Wrap the membrane in Saran Wrap, and store in aluminum foil at 4°C, until ready to perform chemiluminescent detection.

F. Chemiluminescence

Purpose

To enable the detection of the specific DNA-probe reaction.

Reagents

1. Buffer 2: 2% Blocking Reagent in Buffer 1. Combine 768 ml 5% Blocking Reagent (in Buffer 1), 288 ml 4x Buffer 1, and 864 ml dH_2O. Leave 5% Blocking Reagent at room temperature for 10 min before use.
2. Buffer 3: 0.1M Tris-HCl, 0.1M NaCl, 0.05M $MgCl_2$, pH 9.5. Add approximately 1400 ml dH_2O to 200 ml 1M Tris-HCl (pH 9.5) and 50 ml 4M NaCl. Add 100 ml of filter sterilized 1M $MgCl_2$ and mix. Adjust pH to 9.5 and make up to 2 liter with dH_2O. (Note: Do not autoclave, as precipitates tend to form.) Store at room temperature for up to one week.
3. Washing Buffer: 0.3% Tween 20 in Buffer 1. Add 14.4 ml Tween 20 to 1200 ml 4x Buffer 1 and make up to 4800 ml by adding dH_2O.
4. CSPD (Boehringer 1655884): Vortex and centrifuge CSPD in microcentrifuge for 1 min before use. Dilute CSPD stock solution (25mM, 11.6 mg/ml) 1:100 in Buffer 3.
5. Anti-Digoxigenin (DIG)- Alkaline Phosphatase (ALP) Conjugate (Boehringer 1093274): Immediately prior to use, remove anti-DIG-ALP stock conjugate (0.75 U/µl) from the refrigerator, vortex for 15 sec, and centrifuge for 1 min in a microcentrifuge. Make a 1:10,000 dilution of the conjugate in Buffer 2 (i.e., 192 µl of anti-DIG-ALP conjugate in 1920 ml of Buffer 2).

Supplies

1. X-ray film.

Instrumentation

1. Enzyme Box (Boehringer 800058)
2. Platform shaker (Luckham Reciproshake 30)
3. X-ray cassette with intensifying screen
4. X-ray processor

Procedure

All steps are performed at room temperature with shaking using a platform shaker. Use separate enzyme storage boxes for different buffer solutions and keep light-tight. Use one enzyme box for a maximum of three membranes at the same time. It is normal practice in this laboratory for chemiluminescent detection to be performed on 24 membranes at the same time. All membranes are processed up to the end of step 2 (below). Thereafter membranes are processed in groups of six simultaneously, leaving the remaining membranes in the washing buffer.

1. Add 240 ml anti-DIG-ALP conjugate in Buffer 2 to the enzyme box. Place membranes into the boxes DNA side down. Incubate for 15 min on shaker.
2. Transfer membranes to 300 ml of Washing Buffer and incubate for 15 min on shaker. Discard Washing Buffer and replace with fresh Washing Buffer and incubate for an additional 15 min.
3. Transfer membrane to 300 ml of Buffer 3 and incubate for 5 min on shaker.
4. Remove from Buffer 3, place 2 membranes back-to-back in a plastic bag. Add 20 ml of CSPD (1:100 dilution) and reseal the bag. Place the bag on a platform shaker, cover with aluminum foil, and shake for 5 min at room temperature.
5. Pour off CSPD fluid into 20 ml plastic tube for re-use (up to 5 times). Store at -20°C if using on more than 1 day, but note that CSPD should only be frozen once. Carefully remove the membrane from the bag, blot off excess liquid and wrap in Saran Wrap.
6. Tape two membranes to the one X-ray film and place a second film on top. Expose the top film for 5 min and check the intensity of the dots. Depending on these results, process the second film accordingly. It may be necessary to re-expose the membrane to a third or fourth film for a further period of time, depending on dot intensity.

G. Dehybridization

Purpose

To remove probe from membrane thus enabling the membrane to be rehybridized with another probe if required. This method has become redundant in this laboratory since the introduction of the automatic dot blotter, which enables the preparation of sufficient replicate membranes.

Reagents

1. Sodium saline citrate (2x, pH 7.0) (SSC). 0.3 M NaCl + 0.03M tri-sodium citrate.

Procedure

1. Dehybridize a maximum of three membranes in 300 ml of each of the following solutions with shaking:
2. Rinse membranes in dH_2O for 5 min at room temperature.
3. Wash membranes in 0.4M NaOH/0.1% SDS at 45°C for 30 min.
4. Wash membranes in 2x SSC for 30 min at room temperature.
5. Check dehybridization is complete by exposing membranes overnight to X-ray film and developing in usual manner.
6. Store membranes flat at +4°C in a sealed plastic bag if not using immediately.

Results

Record the probe reaction for each sample and analyse according to the known patterns (Tables 9-13) using a computer program. In this laboratory we always have two independent readings of the membrane. We do not believe in recording a result according to the strength of the reaction (e.g., 1, 2, 4, 6, 8, as in serology). The result should be positive or negative. If in doubt, it should be repeated. In the future it would be beneficial to all laboratories if a scanning mechanism was available for reading the membranes, as mistakes are possible in the transmissions of results. We believe it is important that the probe patterns are not analysed by eye. It would be far too easy to see the obvious allele(s) when examining the probe patterns rather that those that are obscure. To overcome this, laboratories should obtain or develop a computer program for the objective analysis of these membranes.

Procedure Notes

When the probe hybridization conditions, i.e., number of picomoles and wash temperature, have been determined, it is worthwhile to keep a record on the performance of the probes. The record should include information such as whether or not the probe gave an adequate signal with its positive control or crossreacted with controls which should be negative. Please note that, on occasions, the conditions for the probes may need to be altered. This in a way is similar to HLA sera, whereby after long term storage the specificities identified can change.

Problem	Try
strong false positive reactions	increasing the wash temperature by 1°C
weak false positive reactions	decreasing the probe concentration by approximately 20%
false negative results	decreasing the wash temperature by 1°C
weak reactions	increasing the probe concentration by approximately 20%

One way to monitor the performance of the probes is to record the length of time needed for autoradiography exposure. If this varies too such an extent that it takes more than 30 minutes to achieve a good signal the conditions of the probe should be altered.

When performing a PCR on 96 samples, there may be one or two samples which are not amplified. Therefore, this laboratory always runs a gel to ensure that there is product. This enables the SSOP method to be well controlled. On some occasions the product appears as a very weak band on the gel; this sample should always be repeated. Good amplification always gives a clean and clear cut SSOP hybridization, while almost all the problematic typing results we have encountered were due to poor amplification. Interpretation of weak hybridization signals can give an incorrect result.

In the methods described each probe is hybridized to two different membranes in the same hybridization bottle and the reagents are prepared accordingly. The SSOP method is thus very suitable for typing large numbers of samples. For example, this laboratory tests 192 samples (96 on each membrane) at the same time, including controls. However, the volume of reagents can be scaled down and if a laboratory is not performing tests on large numbers of samples, only one lot of membranes need be hybridized.

Limitations of the Procedure

As the number of probes is kept to a minimum, not all polymorphic positions are covered. This may lead to the failure to detect new alleles. Despite this, we have discovered 13 new alleles recognized by unique probe patterns. In a medium resolution system, there are many combinations of two alleles giving the same probe pattern as another two alleles. However there are only 8 theoretical combinations at the HLA-A locus which involve alleles from different serological groups. These are listed below along with the number of occasions they presented in 5,000 individuals.

HLA-A Type			**Indistinguishable Type**	
2501	2501	(1)	2501	2603/05
0101	3401	(2)	3601	6601
30	6601		30	2603/05
2501	7401		3201	6601
2501	3201	(10)	3201	2603/05
7401	6601		7401	2603/05
02	6602		0216	3401
02	34	(6)	0203	68

( ) number of occasions this pattern occurred in
5,000 Caucasian individuals from Northern Ireland

There are more of these combinations at the HLA-B locus than at the HLA-A locus. In our population the ones with the highest frequency are: HLA-B*7 cannot be distinguished from HLA-B*81 in the presence of HLA-B*40 (0.9%). HLA-B*15 homozygosity cannot be distinguished from HLA-B*15 present with HLA-B*35 (0.4%); HLA-B*15 cannot be distinguished from HLA-B*35 or HLA-B*53 in the presence of either HLA-B*49 or HLA-B*51 (0.4%).

References

1. Baxter-Lowe LA. HLA-DR and HLA-DQ oligotyping. In: *ASHI Laboratory Manual*, 3rd edition, Nikaein A (Ed.), pIV.C.2.1, 1993
2. Curran MD, Williams F, Earle JAP, Rima BK, Van Dam MG, Bunce M and Middleton D. Long range PCR amplification as an alternative strategy for characterizing novel HLA-B alleles. *Eur. J. Immunogenetics* 23, 297-309, 1996.

Table 1. HLA-A, -B, -C, -DR Primers Used for SSOP Typing

	Primers	Sequence 5'		3'	Band Size
HLA-A		94 (intron 1)	→	116	
GENERIC	A15	GAGGGTCGGGCG(A)GGTCTCAGCCA			863
	AL#AW	TGGCCCCTGGTACCCGT			
		13 (intron 3)	→	274 (exon 3)	
HLA-B		36 (Intron 1)	→	57	
GENERIC	5 BINI-57M	GGGAGGAGC(A)G(A)AGGGGACCGCAG			970
		68 (Intron 3)		37	
	3 BIN3-37M	AGG(C)CCATCCCCGG(C)CGACCTAT			
		68 (Intron 3)	→	37	
	3 BIN3-AC	AGGCCATCCCGGGCGATCTAT			
HLA-B27	5 BINI-57M	see above			970
	3 BIN3-37M	see above			
HLA-C	5 CIN1-61	42 (Intron 1)	→	61	937
GENERIC		AGCGAGGG(T)GCCCGCCCGGCGA			
	3 BCIN3-12	35 (Intron 3)	→	12	
		GGAGATGGGGAAGGCTCCCCACT			
HLA-DRB		(intron1) 15 (exon2)	→	24	
GENERIC	AMP-A*	CCCCACAGCACGTTTCT(C)TG			274
	AMP	CCGCTGCACTGTGAAGCTCT			
		279 (exon2)	→	260	
HLA-DRB					
3/11/6 group	3/11/6 GF	17 (exon2)	→	38	
	AMP-B	GTTTCTTGGAGTACTCTACGTC			263
		CCGCTGCACTGTGAAGCTCT			
		279 (exon2)	→	260	

( ) in primer indicates that at this position two nucleotides are inserted when the primer is being made. The primer is referred to as being degenerate.

Table 2. PCR Master Mixes

Locus	Stock Conc	Master Mix
HLA-A GENERIC		
dH_2O		8220 µl
CRESOL RED	10 mg/ml	100 µl
NH_4 BUFFER	10x	1000 µl
$MgCl_2$	50 mM	300 µl
dNTPs	20 mM each	100 µl
EACH PRIMER (x2)	25 µM	120 µl
TAQ	5 U/µl	40 µl
HLA-B GENERIC		
dH_2O		12345 µl
CRESOL RED	10 mg/ml	150 µl
NH_4 BUFFER	10x	1500 µl
$MgCl_2$	50 mM	450 µl
dNTPs	20 mM each	150 µl
EACH PRIMER (x3)	25 µM	120 µl
TAQ	5 U/µl	45 µl
HLA-B27		
dH_2O		4155 µl
CRESOL RED	10 mg/ml	50 µl
NH_4 BUFFER	10x	500 µl
$MgCl_2$	50 mM	150 µl
dNTPs	20 mM each	50 µl
EACH PRIMER x 2	25 µM	40 µl
TAQ	5 U/µl	15 µl
HLA-C GENERIC		
dH_2O		8300 µl
CRESOL RED	10 mg/ml	100 µl
NH_4 BUFFER	10x	1000 µl
$MgCl_2$	50 mM	300 µl
dNTPs	20 mM each	100 µl
EACH PRIMER x 2	25 µM	80 µl
TAQ	5 U/µl	40 µl
HLA-DRB GENERIC		
dH_2O		8280 µl
CRESOL RED	10 mg/ml	100 µl
NH_4 BUFFER	10x	1000 µl
$MgCl_2$	50 mM	300 µl
dNTPs	20 mM each	100 µl
EACH PRIMER (x2)	25 µM	100 µl
TAQ	50 U/µl	20 µl
HLA-DR3/11/6		
dH_2O		8300 µl
CRESOL RED	10 mg/ml	100 µl
NH_4 BUFFER	10x	1000 µl
$MgCl_2$	50 mM	300 µl
dNTPs	20 mM each	100 µl
EACH PRIMER x 2	25 µM	80 µl
TAQ	5 U/µl	40 µl

Table 3. PCR Amplification Conditions

Locus	Hold	Cycle	No of Cycles	Hold	Hold
HLA-A GENERIC	96°C/5 min	96°C/1 min 60°C/30 sec 72°C/1 min	35	72°C/5 mins	15°C/forever
HLA-B GENERIC + HLA-B27 TESTING	96°C/5 mins	96°C/30 sec 65°C/30 sec 72°C/45 sec	32	72°C/5 mins	15°C/forever
HLA-C GENERIC	96°C/5 mins	96°C/1 min 66°C/30 sec 72°C/1 min	30	72°C/5 mins	15°C/forever
HLA-DRB GENERIC	96°C/5 mins	96°C/1 min 55°C/1 min 72°C/1 min	30	72°C/5 mins	15°C/forever
HLA-DR 3/11/6	96°C/5 mins	96°C/1 min 64°C/1 min 72°C/1 min then 96°C/1 min 56°C/1 min 72°C/1 min	10 20	72°C/5 mins	15°C/forever

Table 4. Probes Used for HLA-A Typing

Probe	Sequence 5'----------3'	Wash Temp (°C)	Picomoles Used	Nucleotide Position
				Exon 2
Z (A89)	GGTATTTCTCCACATCCGT	56	20	17-35
W (A94)	TTCTTCACATCCGTGTC	50	50	22-38
A (56R)	GAGAGGCCTGAGTAT	46	40	163-177
B (62LQ)	TGGGACCTGCAGACA	48	50	178-192
C (62G)	GACGGGGAGACACGG	52	20	181-195
O (62RN)	GACCGGAACACACGG	52	20	181-195
D (62EG)	GAGGAGACAGGGAAA	46	40	184-198
Y (A276)	GGCCCACTCACAGACT	52	50	204-219
E (731)	TCACAGATTGACCGA	45	40	211-225
X (A290)	CTGACCGAGTGGACCT	51	40	218-233
R (A26)	TGACCGAGCGAACCTG	54	40	219-234
F (77S)	GAGAGCCTGCGGATC	50	20	226-240
				Exon 3
Z (A347)	CTCACACCATCCAGA	45	70	5-19
T (95V)	CACACCGTCCAGAGG	48	40	7-21
P (114EH)	TATGAACAGCACGCC	46	30	67-81
G (131R)	CGCTCTTGGACCGCG	52	40	121-136
H (142TK)	ACCACCAAGCACAAG	46	40	154-168
I (149T)	TGGGAGACGGCCCAT	50	40	169-183
J (150V)	GAGGCGGTCCATGCG	60	20	172-186
K (151R)	GCGGCCCGTGTGGCG	60	20	175-189
1 (A525)	TGAGGCGGAGCAGTTG	54	40	183-198
N (156Q)	GAGCAGCAGAGAGCC	52	20	190-204
Q (156W)	GAGCAGTGGAGAGCC	50	10	190-204
L (161D)	CTGGATGGCACGTGC	50	20	208-222
V (A551)	TGGAGGGCACGTGCGT	56	40	209-224
M (163R)	GAGGGCCGGTGCGTG	54	20	211-225
S (A355)	GGCGAGTGCGTGGAGTGGC	68	10	214-232
U (A357)	GGCGAGTGCGTGGACGGGC	68	10	214-232

Table 5. Probes Used for HLA-B Typing

Probe	Sequence 5'----------3'	Wash Temp (°C)	Picomoles Used	Nucleotide Position
				Exon 2
31 (B89)	GGTATTTCGACACCGCC	56	40	17-33
32 (B156)	GGACGGCACCCAGTT	52	40	84-98
33 (B168)	GTTCGTGCGGTTCGA	50	40	96-110
09 (BL09)	GAGTCCGAGAGAGGAGCC	57	6	123-140
01 (BL01)	GAGGAAGGAGCCGCGGGC	64	20	129-146
02 (BL02)	GAGGACGGAGCCCCGGGC	64	40	129-146
07 (BL07)	GAGGATGGCGCCCCGGGC	64	60	129-146
34 (B249)	TTGGGACGGGGAGAC	50	40	177-191
24 (BL24)	GGGAGACACAGATCTCCA	55	40	185-202
05 (BL05)	ACACAGATCTTCAAGACC	55	14	190-207
10 (BL10)	GATCTACAAGGCCCAGGC	58	10	195-212
12 (BL12)	ATCTGCAAGGCCAAGGCA	56	20	196-213
18 (BL18)	ACTGACCGAGTGAGCCTG	58	20	217-234
35 (B73)	ACTGACCGAGTGGGCCTG	63	40	217-234
20 (BL20)*	AGCGGAGCGCGGTGCGCA	64	40	233-250
21 (BL21)	CGGAACCTGCGCGGCTAC	62	40	235-252
22 (BL22)	CGGACCCTGCTCCGCTAC	61	30	235-252
23 (BL23)	CGGATCGCGCTCCGCTAC	62	40	235-252
				Exon 3
27 (BL27)	CTCACACTTGGCAGAGGA	56	20	5-22
36 (B348)	TCACACCATCCAGAGG	49	50	6-21
37 (B354)	CATCCAGGTGATGTAT	46	40	12-27
28 (BL28)	CCAGTGGATGTATGGCTG	56	40	15-32
38 (B361)	AGGATGTTTGGCTGC	48	40	19-33
26 (BL26)	CTGCGACCTGGGGCCCGA	65	40	30-47
30 (BL30)	GGCATAACCAGTTAGCCT	54	50	65-82
39 (B409)	TATGACCAGGACGCCT	55	40	67-82
40 (B427)	GACGGCAAAGATTACA	46	40	85-100
41 (B499)	ACCCAGCTCAAGTGG	47	40	157-171
42 (B505)	CGCAAGTTGGAGGC	46	40	163-176
43 (B532)	GAGCAGCTGAGAGCCT	52	40	190-205
44 (B539)	GAGAACCTACCTGGA	46	40	197-211
45 (B553a)	GAGGGCCTGTGCGT	48	40	211-224
46 (B553b)	GAGGGCACGTGCGT	48	40	211-224
47 (B566)	TGGAGTCGCTCCGC	48	40	224-237
48 (B597)	GAAGGACACGCTGGA	52	40	255-269
49 (B599)	AGGACAAGCTGGAGCG	52	40	257-272

* = complementary to coding sequence

Table 6. Probes Used for HLA-C Typing

Probe	Sequence 5'----------3'	Wash Temp (°C)	Picomoles Used	Nucleotide Position
				Exon 2
15 (C2D6)	AGCCCCGGGCGCCGT	56	35	137-151
1 (C2EALL)	GGGTGGAGCAGGAGGG	56	20	152-167
3 (C2G2)	AGTGAACCTGCGGAAACTG	59	25	225-243
2 (C2G1)	TGAGCCTGCGGAACCTG	56	30	227-243
17 (C2H303)	CCAGAGCGAGGCCAGT	54	25	258-2 (intron 2)
				Exon 3
21 (C3A14)	CTCCAGTGGATGTTTGGC	56	25	13-30
4 (C3A1)	TCCAGTGGATGTGTGGC	54	25	14-30
19 (C3A4)	CAGAGGATGTTTGGCTGC	56	20	16-33
12 (C3A7023)	AGGATGTCTGGCTGCGA	54	25	19-35
7 (C3A212)	TGTACGGCTGCGACCTG	56	20	23-39
22 (C3C15)	GGCATGACCAGTTAGCC	54	25	65-81
18 (C3CA)	GTATGACCAGTCCGCCT	54	25	66-82
20 (C3D58)	GCCCTGAATGAGGACCT	55	30	103-119
6 (C3E1203)	TCCTGGACTGCCGCGG	56	25	124-139
5 (C3E12)	GGACCGCTGCGGACAC	56	25	128-143
11 (C3G17712)	CGCAAGTTGGAGGCGG	54	25	163-178
13 (C3G716)	GGCCCGTGCGGCGGA	56	25	177-191
23 (C3G8013)	GCCCGTACGGCGGAG	54	25	178-192
8 (C3G2612)	TGAGGCGGAGCAGTGGA	57	25	183-199
14 (C3G16)	GCGGCGGAGCAGCAGA	57	25	184-199
9 (C3H2)	GGAGGGCGAGTGCGTG	57	25	210-225
16 (C3H3)	GGAGGGCCTGTGCGTG	56	25	210-225
10 (C3J17)	GCTCCGCGGATACCTG	54	25	231-246

Table 7. Probes used for HLA-DR typing

Probe	Sequence 5'----------3'	Wash Temp (°C)	Picomoles Used	Nucleotide Position
				Exon 2
09 (1007)	GAAGCAGGATAAGTTTGA	50	10	24-41
03 (1008N)	GAGGAGGTTAAGTTTGAG	54	2	25-42
07 (1004)	GAGCAGGTTAAACATGAG	56	4	25-42
08 (1006)	TGGCAGGGTAAGTATAAG	50	10	25-42
06 (1003)	GTACTCTACGTCTGAGTG	56	4	27-44
02 (1002)	AGCCTAAGAGGGAGTGTC	56	30	29-46
18 (DR18)	CTACGGGTGAGTGTTAT	48	40	32-48
10 (2810)	GCGAGTGTGGAACCTGAT	56	10	66-83
01 (2801)	CGGTTGCTGGAAAGATGC	60	6	73-90
25 (DR25)	CGGTTCCTGGACAGATA	52	40	73-89
11 (DRB12)	CAGGAGGAGCTCCTGCGC	58	4	100-117
13 (DRB6)	CAGGAGGAGAACGTGCG	62	7	100-117
22 (DR22)	CCGGCCTAGCGCCGAGTA	58	50	163-179
05 (5703)	GCCTGATGAGGAGTACTG	54	20	165-182
15 (DRB14/1)	GGCCTGCTGCGGAGCACT	64	4	164-181
26 (DRB ALL)	TGGAACAGCCAGAAGGAC	56	40	181-198
14 (7031)	CTGGAAGACAAGCGGGCCG	60	30	202-220
16 (DRB13)	TGGAAGACGAGCGGGCCG	64	3	203-220
27 (DR27)	TGGAGCAGGCGCGG	50	20	203-216
28 (DR28)	AGACAGGCGCGCCG	52	20	207-220
24 (DR24)	AGCGGAGGCGGGCCGAG	62	40	206-222
17 (7012) *	ACCGCGGCCCGCCTCTGC	66	30	207-224
23 (7005) *	ACCGCGGCCCGCTTCTGC	66	40	207-224
12 (DRB8)	GCGGGCCCTGGTGGACAC	64	20	213-230
04 (7004)	GGCCGGGTGGACAACTAC	62	1	17-234

* = complimentary to coding sequence

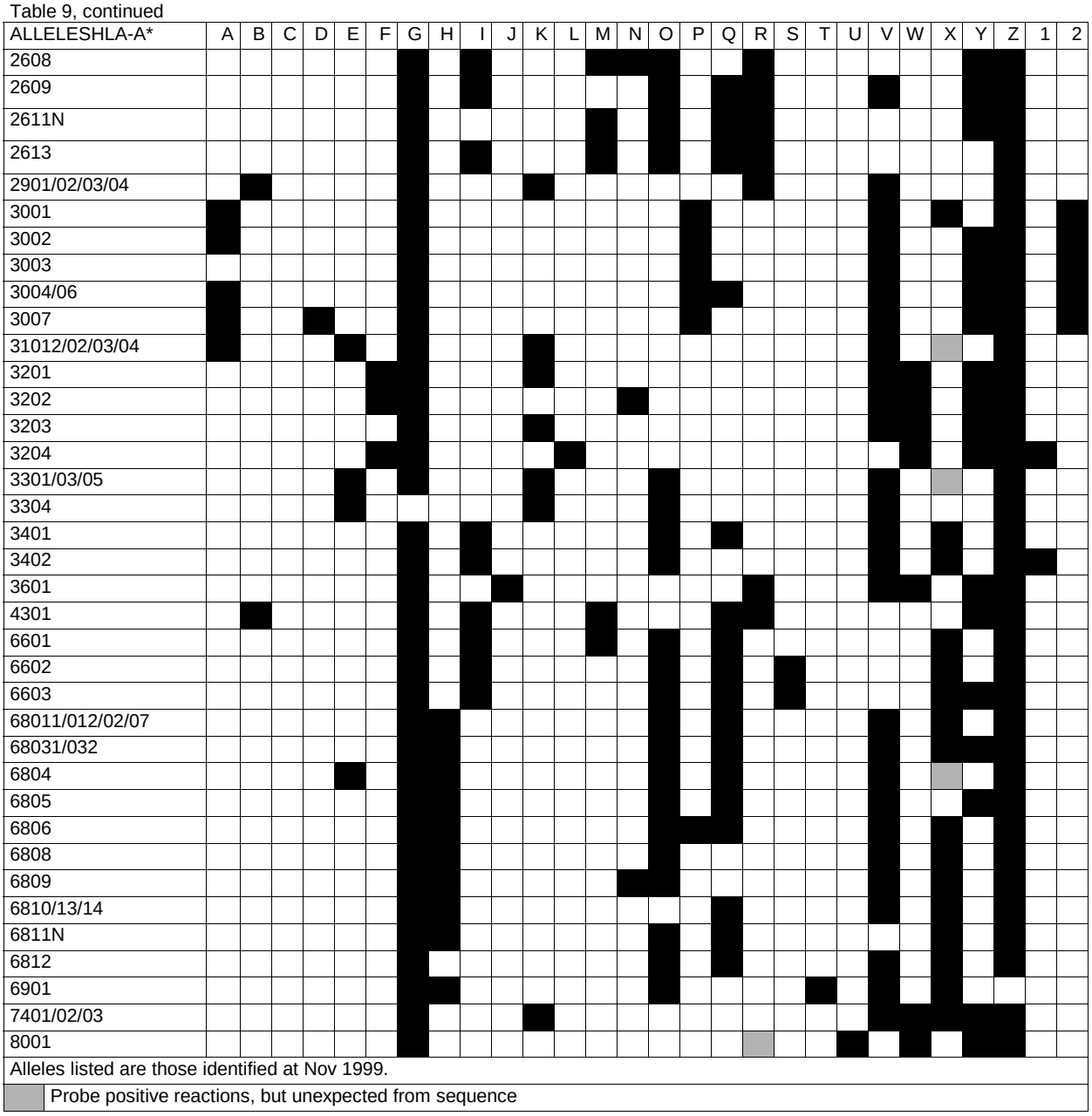
Table 8. Probes Used for HLA-DR 3/11/6 Group

Probe	Sequence 5'----------3'	Wash Temp (°C)	Picomoles Used	Nucleotide Position
				Exon2
1 (DR19)	CGGTACCTGGACAGAT	50	40	73-88
2 (5703)	GCCTGATGAGGAGTACTG	54	20	165-182
3 (DRB14/1)	GGCCTGCTGCGGAGCACT	64	4	164-181
4 (7031)	CTGGAAGACAAGCGGGCCG	60	30	202-220
5 (DRB13)	TGGAAGACGAGCGGGCCG	64	3	203-220
6 (DR24)	AGCGGAGGCGGGCCGAG	62	40	206-222
7 (7012)	ACCGCGGCCCGCCTCTGC	66	30	207-224
8 (7005)	ACCGCGGCCCGCTTCTGC	65	40	207-224
9 (DRB8)	GCGGGCCCTGGTGGACAC	64	20	213-230
10 (7004)	GGCCGGGTGGACAACTAC	62	1	217-234
11 (5701)	GCCTGATGCCGAGTACTG	58	40	165-182

TABLE 9: HLA-A SSOP PATTERNS

Probes	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y	Z	1	2
HLA-A* Alleles																												
0101/03/04N/05N/06							■			■			+					■					■		■	■		
0102							■			■			+					■							■	■		■
02011/012/07/09/13/ 15N/18/20/24/29/30/ 32N/33			■				■	■												■		■	■		■			
0202			■				■	■									■					■			■			
0203			■				■	■	■								■			■		■			■			
0204/171/172			■				■	■														■			■			
0205/08			■				■										■					■			■			
0206/10/21/28			■				■													■		■			■			
0211			■		■		■													■		■	■					
0212/13/27			■				■							■						■		■	■		■			
0214			■				■															■			■			
0216			■				■	■											■	■			■		■			
0219			■				■							■						■		■	■		■			
0222			■				■	■									■			■		■	■		■			
0225			■				■	■	■											■		■	■		■			
0226			■				■	■												■		■	■		■		■	
0234			■				■	■												■		■	■					
0235			■				■	■												■		■	■	■				
03011/013/03N/04							■					■											■	■		■	■	
03012							■					■											■	■		■		
0302							■					■		■									■	■		■		
11011/012/02/03/05							■						■	■										■		■		
1104							■							■									■	■		■		
2301				■			■				■											■			■			■
2302				■			■				■						■					■			■			■
2402101/102L/031/ 032/05/09N/11N/15/17				■			■							■								■			■			■
2404				■			■							■				■				■			■			■
2406				■			■										■					■			■			■
2407				■			■							■								■						■
2408			■				■							■								■			■			■
2410				■			■						■	■														■
2413/22				■			■															■			■			■
2414				■			■							■						■		■			■			■
2416				■			■				■											■			■	■		
2418				■			■					■													■		■	
2424							■				■				■							■						■
2501						■	■		■				■		■		■								■	■		
2502						■	■		■				■		■		■									■		
2503						■	■		■				■		■		■						■		■	■		
2601/02/10/12							■		■				■		■		■	■							■	■		
2603/05/06							■		■				■		■		■								■	■		
2604							■		■						■		■	■							■	■		
2607			■				■		■				■				■	■							■	■		

Table 9, continued



ALLELESHLA-A*	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y	Z	1	2
2608							■		■				■	■	■			■							■	■		
2609							■		■						■		■	■				■			■	■		
2611N							■						■		■		■	■							■	■		
2613							■		■				■		■		■	■								■		
2901/02/03/04		■					■				■							■				■				■		
3001	■						■									■						■		■		■		■
3002	■						■									■						■			■	■		■
3003							■									■						■			■	■		■
3004/06	■						■									■	■					■			■	■		■
3007	■			■			■									■						■			■	■		■
31012/02/03/04	■				■		■				■											■				■		
3201						■	■				■											■	■		■	■		
3202						■	■							■								■	■		■	■		
3203							■				■											■	■			■		
3204						■	■					■													■	■	■	
3301/03/05					■		■				■				■							■			■			
3304					■						■				■							■						
3401							■		■						■		■					■			■			
3402							■		■						■							■			■		■	
3601							■			■								■				■	■			■		
4301		■					■		■				■				■	■								■		
6601							■		■				■		■		■								■			
6602							■		■						■		■		■									
6603							■		■						■		■		■							■		
68011/012/02/07							■	■							■		■					■						
68031/032							■	■							■		■					■			■	■		
6804					■		■	■							■		■					■				■		
6805							■	■							■		■					■			■			
6806							■	■							■	■	■					■						
6808							■	■							■							■			■			
6809							■	■						■	■							■			■			
6810/13/14							■	■									■					■			■			
6811N							■	■							■		■								■			
6812							■								■		■					■			■			
6901							■	■							■					■		■			■			
7401/02/03							■				■											■	■	■	■	■		
8001							■														■		■		■	■		

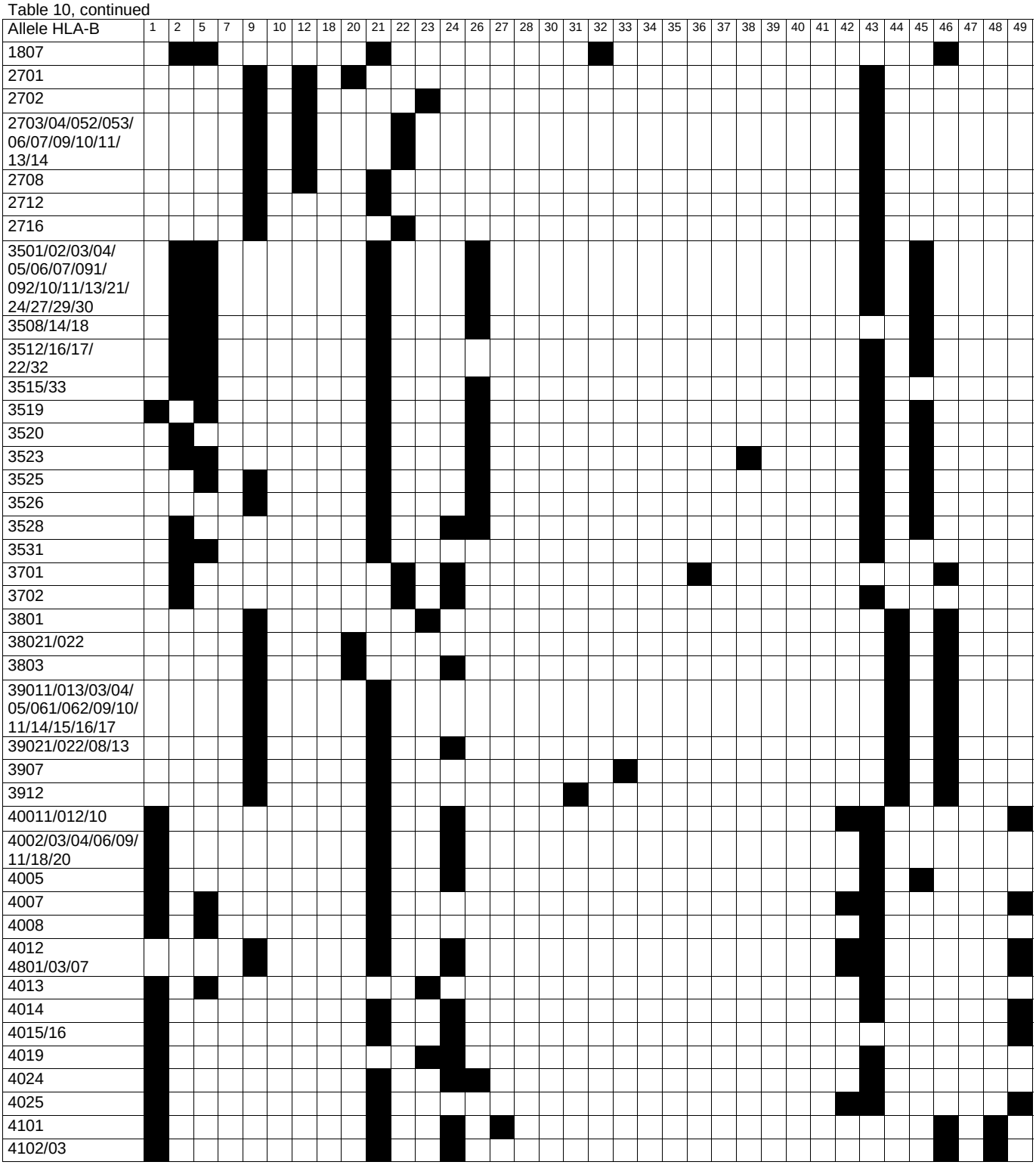
Alleles listed are those identified at Nov 1999.

Probe positive reactions, but unexpected from sequence

TABLE 10 HLA-B SSOP PATTERNS

Probes	1	2	5	7	9	10	12	18	20	21	22	23	24	26	27	28	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49
HLA-B* Alleles																																				
07021/022/023/ 04/05/06/07/09/11					■	■				■																										■
0703/10/16					■					■																										■
0708			■		■					■																										■
0712					■	■				■				■																						■
0713										■																										■
0801/06/07/09/08/ 2010			■		■					■								■														■		■	
0802			■		■				■									■														■		■	
0803			■		■							■						■														■		■	
0804/05					■					■								■														■		■	
1301/02				■					■				■				■										■		■						
1303				■					■				■				■												■		■				
1304, 1536				■					■				■																■		■				
1401/02/04					■					■						■													■			■			
1403					■					■						■																■			
1405/062					■					■																			■			■			
1501101/102N/ 04/07/12/19/26N/ 30/32/33/34/35/ 38/45/57				■						■			■																	■					
15012				■						■			■														■				■				
1502/21/31				■						■																			■		■				
1503					■					■			■																■		■				
1505/25/39				■						■			■																■		■				
1506				■						■			■												■				■		■				
1508			■	■						■																					■				
1509					■					■																	■			■		■				
1510/18/37					■					■																				■		■				
1511/15/28				■						■																						■				
1513/17				■								■																		■		■				
1514				■						■			■																			■		■		
1516				■								■			■															■		■				
1520				■						■			■	■																■		■				
1522		■	■							■																						■				
1523					■							■																		■		■				
1524				■								■	■																			■				
1527				■						■			■												■							■				
1529			■		■					■																				■		■				
1540				■						■			■																	■						
1542				■						■			■				■													■			■			
1543				■							■		■																	■		■				
1544				■						■																				■			■			
1546	■									■			■																			■				
1547/49					■					■			■																							
1548				■						■			■																		■	■				
1550				■						■			■																				■			
1551					■					■																						■				
1801/02/03/04/05/ 06		■								■									■										■			■				

Table 10, continued



Allele HLA-B	1	2	5	7	9	10	12	18	20	21	22	23	24	26	27	28	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49
1807		■	■							■									■													■			
2701					■		■		■																					■					
2702					■		■					■																		■					
2703/04/052/053/ 06/07/09/10/11/ 13/14					■		■				■																			■					
2708					■		■			■																				■					
2712					■					■																				■					
2716					■						■																			■					
3501/02/03/04/ 05/06/07/091/ 092/10/11/13/21/ 24/27/29/30		■	■							■				■															■		■			
3508/14/18		■	■							■				■																	■			
3512/16/17/ 22/32		■	■							■																			■		■			
3515/33		■	■							■				■															■					
3519	■		■							■				■															■		■			
3520		■								■				■															■		■			
3523		■	■							■				■										■					■		■			
3525			■		■					■				■															■		■			
3526					■					■				■															■		■			
3528		■								■			■	■															■		■			
3531		■	■							■																			■					
3701		■									■		■										■									■			
3702		■									■		■																■					
3801					■							■																		■		■			
38021/022					■				■																					■		■			
3803					■				■				■																	■		■			
39011/013/03/04/ 05/061/062/09/10/ 11/14/15/16/17					■					■																				■		■			
39021/022/08/13					■					■			■																	■		■			
3907					■					■										■										■		■			
3912					■					■								■												■		■			
40011/012/10	■									■			■																■	■					■
4002/03/04/06/09/ 11/18/20	■									■			■																	■					
4005	■									■			■																	■		■			
4007	■		■							■																			■	■					■
4008	■		■							■																				■					
4012 4801/03/07					■					■			■																■	■					■
4013	■		■									■																		■					
4014	■									■			■																	■					■
4015/16	■									■			■																						■
4019	■											■	■																	■					
4024	■									■			■	■																■					
4025	■									■																			■	■					■
4101	■									■			■		■																	■		■		
4102/03	■									■			■																			■		■		

Table 10, continued

Allele HLA-B	1	2	5	7	9	10	12	18	20	21	22	23	24	26	27	28	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49
4201/02					■	■				■																							■		■	
4402	■								■				■													■						■		■		
44031/032/07/13	■								■				■													■				■		■		■		
4404	■								■				■													■							■	■		
4405/14	■								■				■																			■		■		
4406		■	■									■														■						■		■		
4408				■					■				■													■						■		■		
4409	■									■			■													■						■		■		
4410	■								■				■	■			■								■					■		■		■		
4411	■												■													■						■		■		
4412	■								■																	■						■		■		
4415	■								■				■	■	■																	■		■		
4501/02	■									■			■	■	■																	■		■		
4601				■				■		■																						■				
4701	■										■		■												■					■						
4702	■									■			■												■					■						
4703	■												■												■					■						
4802					■					■			■	■																■		■				
4804					■					■			■																■	■					
4805					■					■			■																							■
4806			■		■					■																			■	■					■
4901	■											■	■	■	■															■		■				
5001	■									■			■	■	■															■		■				
5002	■									■			■	■	■															■		■		■		
5004	■									■			■		■															■		■				
51011/012/021/ 022/03/04/06/ 09/11N/14/17		■	■									■														■			■		■				
5105/08		■	■									■														■					■				
5107		■										■														■			■		■				
5110		■	■									■																	■						
5112		■	■																							■			■		■				
5113		■	■									■																	■		■				
5115		■	■									■					■												■		■				
5116		■	■									■															■		■						
5119		■	■									■															■			■	■	■				
52011/012		■										■	■														■			■		■				
5301/02/04		■	■									■		■																■		■				
5303		■	■								■			■																■		■				
5401						■				■							■			■										■			■			
5501/02/05					■	■				■							■													■			■			
5503					■	■		■		■							■													■			■			
5504					■	■				■																				■			■			
5507						■				■							■													■			■			
5508					■	■				■																				■		■				
5601/02/04					■	■				■							■													■		■				
5603					■	■				■																						■				
5605					■	■				■																	■			■		■				

Table 10, continued

Allele HLA-B	1	2	5	7	9	10	12	18	20	21	22	23	24	26	27	28	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49
5701/03				■								■									■			■						■		■					
5702				■								■									■			■								■					
5704				■								■									■			■		■						■					
5705				■								■		■							■											■					
5801		■										■		■							■									■		■					
5802		■										■		■		■					■									■		■					
5901			■		■							■					■													■			■				
67011/012					■	■				■																					■		■			
7301					■		■			■												■								■							
7801/021/022/04		■	■							■																	■			■		■				
7803		■								■																	■			■		■				
8101					■	■				■																			■	■						■
8201										■				■			■								■									■			

Alleles listed are those identified at Nov 1999.

Table 11: HLA-C SSOP Patterns

Probes	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
HLA-Cw* Alleles																							
0102/03	■	■		■																			
0104	■	■		■	■			■										■					
02021	■						■	■	■									■					
02022/024	■		■				■	■	■									■					
02023	■		■					■	■									■					
0203	■		■				■		■									■					
03031/032/11	■	■														■	■						
0302	■	■														■		■					
03041/042/05/08/06/2009	■	■														■							
0307	■		■													■							
04011/012/03/04/05/06/07	■		■																■				
0501/02	■		■																	■			
0602/03	■		■					■							■			■					
0604	■		■												■			■					
07011/05/06	■	■									■	■	■					■					
0702/10	■	■									■	■	■					■					
0703	■	■										■	■					■					
0704/11	■	■									■		■		■								
0707/09	■		■								■		■					■					
0708	■	■									■		■					■	■				
0712	■	■											■		■								
0713	■	■									■	■	■										
0714	■	■					■				■		■					■					
0801/03/06	■	■																		■			■
0802/04/05	■	■																		■			
12021/022	■	■			■		■	■										■					
1203/06	■	■				■		■										■					
12041	■		■					■										■					
12042/05	■		■			■		■										■					
1301	■	■			■		■	■							■			■					
14021/022/03	■	■																■			■		
1404	■																	■			■		
15051/052/06	■		■																				
15021/03/08	■		■																			■	
1504	■		■															■					
1507	■	■																				■	
1601	■	■											■	■				■					
1602	■		■										■	■				■					
16041	■	■											■					■					
1701/02	■		■						■	■	■												
1801/02	■		■												■				■				

Alleles listed are those identified at Nov 1999.

TABLE 12: HLA-DR SSOP PATTERNS																									
Probes	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	22	23	24	25	26	27	28
HLA-DR* Alleles																									
0101/021/04/05	■																■						■		
01022	■																						■		
0103	■															■							■		
0106	■																						■	■	
03011/021/03/06/05/2013				■		■							■										■		
03012/022/11/14/15						■							■										■		
0304/09				■		■																	■		
0307				■		■							■									■	■		
0308				■	■	■							■										■		
0310				■		■							■		■								■		
0312				■		■							■						■				■		
04011/012/13/16/21/26/33							■			■										■	■	■	■		
0402/14							■			■						■					■	■	■		
0404/08/19/23							■			■							■				■	■	■		
04051/052/10/28/29/30							■			■							■		■		■	■	■		
04031/032/06/07/20/27/32							■			■											■	■	■		
0409							■			■									■	■	■	■	■		
0411/17/24							■			■									■		■	■	■		
0412							■			■		■							■		■	■	■		
0415					■		■			■											■	■	■		
0418/25/31							■			■		■									■	■	■		
0422				■			■			■											■	■	■		
07011/03/04								■		■											■		■		
07012										■											■		■		
0801/032/06/10/12/16/17												■						■	■			■	■		
08021/022/041/042/043/ 07/09/11/13/15/19 1415												■						■				■	■		
0805/18																		■	■			■	■		
0808												■			■			■				■	■		
0814												■							■			■	■		
0820						■						■										■	■		
0821												■										■	■		
09012									■	■											■		■		
1001			■																				■		
11011/012/041/042/06/081 /082/10/12/13/15/18/19/24/ 27/28/29/32					■	■																■	■		
11013					■	■																■	■		■
1102/03/11/14/21					■	■										■						■	■		
1105/30					■																	■	■		
1107				■	■	■																■	■		
1109					■	■																■	■		

TABLE 12, continued

HLA-DR* Alleles	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	22	23	24	25	26	27	28
1116/20					■	■							■			■						■	■		
1117					■																■	■	■		
1122					■		■															■	■		
1123/25					■	■						■										■	■		
1126/34					■	■											■					■	■		
1131/33/35 13071/072/11/14/25						■																■	■		
1201/021/022/032/06											■							■					■		■
1204					■						■							■					■		■
1205																		■					■		■
1301/02/16/20/28/29/31/35						■							■			■						■	■		
13031/032/33						■								■					■			■	■		
1304						■										■			■			■	■		
1305/06 1433						■							■									■	■		
1308/22/23/24/34						■										■						■	■		
1309						■							■									■	■	■	
1310						■							■	■								■	■		
1312/21/30						■													■			■	■		
1313						■						■							■			■	■		
1315/27						■							■			■							■		
1317																■		■				■	■		
1318						■						■	■									■	■		
1319						■										■							■		
1326						■							■										■		■
1332						■							■			■			■			■	■		
1401/07						■									■						■	■	■		
1402/06/29						■							■				■								
1403/12/27						■						■	■												
1404/28															■			■				■	■		
1405/08/14/23						■																■	■		
1409/17/30						■							■				■						■		
1410							■								■							■	■		
1411					■													■				■	■		
1413						■							■				■		■						
1416						■									■	■						■			
1418						■							■								■				
1419						■							■							■					
1420						■											■								
1421						■							■							■		■			
1422/25/32						■									■							■			
1424						■							■											■	
1426						■									■						■				
1431															■			■				■	■		
15011/012/021/022/023/ 04/05/06/07 16011/012/021/022/03/05		■																				■	■	+	+
1503/1607		■																						+	+
1508		■																				■			
1604		■										■										■	■	+	+
1608		■											■									■	■	+	+
	Alleles listed are those identified at Nov 1999																								

TABLE 13 : HLA-DR 3/11/6 SUBGROUP PATTERNS											
Probes	1	2	3	4	5	6	7	8	9	10	11
HLA-DR* Alleles											
03011/04/05/06/09	■									■	■
03012/11/14/15	■										■
03021/03/07										■	■
0308	■	■								■	
0310	■		■							■	
0312/13	■									■	
11011/012/013/041/042/06 /081/082/09/10/12/13/15/1 8/19/24/27/28/29/32		■									
1102/03/11/14/16/20/21		■			■						
1107		■								■	
1117		■				■					
1123/25		■							■		
1126/34		■					■				
1301/02/08/15/16/19/20/22 /23/24/28/29/34/35					■						■
13031/032/33				■							
1304/31/32					■						
1305/06/09/11/14/25 1424/33 03022/071											■
13072/12/21/26/30											
1310				■							■
1313									■		
1318 1403/12/27 0820									■		■
1327	■				■						■
1401/07/26			■			■					
1402/06/09/17/20/29/30							■				■
1405/08/18						■					
1413							■				
1414/23						■					■
1416			■		■						
1419/21								■			■
1422/25/32			■								
Alleles listed are those identified at Nov 1999.											
ALLELES NOT AMPLIFIED											
1105 1122 1130 1317 1404 1410 1411 1415 1428 1431											
All 8's except 0820											

HLA-DPA1 and -DPB1 Typing Using the Polymerase Chain Reaction and Non-Radioactive Sequence-Specific Oligonucleotide Probes

Lori L. Steiner, Priscilla V. Moonswamy, Teodorica L. Bugawan, and Ann B. Begovich

Purpose

The HLA-D region contains many genes which encode polypeptides that play a central role in the regulation of the immune process. Within the HLA-D region there are three sub-regions, HLA-DR, -DQ, and -DP, which contain the classical class II genes. A great deal is known about the structure and function of the HLA-DR and -DQ molecules, but, until recently, relatively little was known about the DP molecule and the genes encoding it. This is most likely due to the low levels of cell surface expression of the DP molecule, making it difficult to generate DP-specific serologic reagents. In addition, because of this low level of expression, the DP molecule also appears to elicit a weak response in the primary mixed lymphocyte reaction (MLR).[1] Consequently, the two techniques which were invaluable in the initial characterization of the variability in the DR and DQ molecules proved ineffective for DP.

The development of the polymerase chain reaction (PCR)[2] has revolutionized the field of molecular biology and, when used with other techniques, has permitted a detailed characterization of multiple genes and gene families in a relatively short period of time. One such area in which this technique has proved extremely useful is the characterization of the genes encoding the HLA molecules, including DPA1 and DPB1. Prior to the development of this technology, the standard method for DP typing was the primed lymphocyte typing (PLT) assay.[3-5] This cellular assay, which was time-consuming, difficult to perform, and relied on specifically primed T-cells, detected only six different DP specificities, DPw1-DPw6. Other cellular,[6] biochemical,[7] and restriction fragment length polymorphism (RFLP)[8] analyses suggested these six specificities were an underestimate of the actual degree of polymorphism within DP.

Using the PCR to amplify genomic DNA and cDNA and a variety of different techniques to characterize the resulting PCR product, we now know that both the DPA1 and DPB1 molecules are highly variable. To date, the nucleotide sequences of 77 DPB1 and 11 DPA1 alleles have been reported.[9,10] At the DPB1 locus, 72 of these 77 alleles encode unique amino acid sequences while the remaining five encode silent nucleotide changes. Comparison of these DPB1 sequences reveals an unusual pattern of polymorphism; DPB1 variation is almost exclusively localized to 18 amino acid residues within six regions of variability in the first extracellular domain of the protein (which is encoded by the second exon of the DPB1 gene). In addition, the majority of the nucleotide substitutions observed in the second exon are non-synonymous amino acid replacement changes. Within each region of variability, which can range from one to five amino acids in length, there are: a) a limited number of polymorphic residues (n = 2-4) at each amino acid position, and b) between three and six common polymorphic sequence motifs, few of which are allele-specific. Instead, the shuffling of these limited numbers of sequence motifs in the six regions of variability results in the formation of the various alleles. This shuffling of sequence motifs, which leads to a "patchwork" pattern of polymorphism, is characteristic of the DPB1 locus.

Of the 11 DPA1 alleles, eight encode unique amino acid sequences, while the remaining three contain silent nucleotide substitutions. In addition to being less diverse, the second exon of the DPA1 locus also contains more silent (synonymous) nucleotide changes than found in DPB1; only nine amino acid positions are variable in the first domain of DPA1 compared to 18 in the first domain of DPB1. At each of these nine residues only two amino acids have been observed.

A variety of different populations have now been typed for the DPA1 and DPB1 loci.[11-13] The results show that, in most populations, there is one predominant DPA1 and DPB1 allele; the identity of this common allele is dependent on the ethnic origin of the population. The DP molecule has also been shown to play a role in susceptibility to certain autoimmune disorders including pauciarticular juvenile rheumatoid arthritis,[14-17] type I diabetes,[18, 19] and chronic beryllium disease, an environmentally-induced lung disorder.[20, 21] Together, these observations suggest that the DP molecule may be more important functionally than originally thought. Consequently, methods for DPA1 and DPB1 typing using the PCR and non-radioactive sequence-specific oligonucleotide probes (SSOPs) have been developed and are described in this procedure.

Specimen

A. Preparation of Genomic DNA

In order to obtain the best PCR amplification results, one should start with a pure sample of genomic DNA. There are many kits and methods available for purifying DNA, and we recommend any one of the following: PureGene DNA Isolation Kit (Gentra Systems, Minneapolis, MN, USA), QIAamp Blood Kit (QIAGEN, Santa Clarita, CA, USA), or standard phenol-chloroform extraction of genomic DNA.[22]

B. Control DNAs

To ensure that each probe has the correct specificity and that the assay is performed and interpreted correctly, a no-DNA control as well as a positive control DNA for each probe must be included in every assay. (A positive control for one probe in a region serves as a negative control for another probe within the same region.) Either genomic DNA or DNA from cloned PCR product can be used. In this laboratory, we use DNA isolated from the following B lymphoblastoid cell lines:

1. DPA1-Typing:

Cell Line	DPA1 Type
LBUF	DPA1*02011
CB6B	DPA1*02021
AMAI	DPA1*0301
T7526	DPA1*0401
SK*	DPA1*0104

*We do not have an available cell line with the DPA1*0104 allele; consequently we have cloned and purified the second exon of DPA1*0104 from a DNA sample, SK, carrying this allele to use as a source of control DNA.

2. DPB1-Typing:

Cell Line	DPB1 Type
LKT3	DPB1*0501
LBUF	DPB1*1701
TER81	DPB1*0101,*1301
JY	DPB1*0201,*0401
BIN40	DPB1*0301,*0601
PLH	DPB1*1501
CRK	DPB1*01011*11011
AH696*	DPB1*11012
NG78*	DPB1*3201
SE53*	DPB1*3801
T93*	DPB1*4101
C23*	DPB1*6001
C53*	DPB1*6101N

*We do not have available cell lines with the DPB1*11012, *3201, *3801, *4101, *6001, and *6101N alleles. Consequently, we have cloned and purified the DPB1 second exons from DNA samples carrying these alleles to use as sources of control DNAs.

Reagents and Supplies

A. DP Amplifications

1. Micropipettes (designated clean) and aerosol resistant micropipette tips for PCR setup (Rainin, Emeryville, CA, USA). These pipettes should be used exclusively for PCR setup and should not come into contact with PCR-product or any reagents exposed to PCR-product.
2. A 2X PCR premix containing 100 mM KCl, 20 mM Tris-HCl (pH 8.3), 30% glycerol, 0.375 mM dTTP, 0.375 mM dGTP, 0.375 mM dCTP, 0.375 mM dATP, 3 mM $MgCl_2$, and 0.1 U/µl *Taq* polymerase. Fifty µl of this 2X premix will be used for each 100 µl amplification reaction. This premix should be prepared in a designated clean area. It can be made in bulk and stored at 2-8°C until use.
3. DPA1 locus-specific primers DPA1-F: 5′ACATTTTGTCGTGTTTTTCTCTA3′ and DPA1-R: 5′ GAAGGTCAACCCGATGTC3′ (Rozemuller et. al., in preparation), each at 50 µM. These primers are intronic and flank exon 2.
4. DPB1 locus-specific primers UG19: 5′ GCTGCAGGAGAGTGGCGCCTCCGCTCAT 3′ and UG21: 5′ CGGATCCGGCCCAAAGCCCTCACTC 3′,[23] each at 50 µM. These primers are intronic and flank exon 2.
5. DPA1 group-specific primer: see Table 1.
6. DPB1 group-specific primers: see Table 1.
7. Thermocycler: GeneAmp PCR System 9600 or GeneAmp PCR System 2400 (Perkin Elmer, Norwalk, CT, USA).

B. Gel Electrophoresis

1. 10X TB: 0.89 M Tris, 0.89 M boric acid, 0.025 M $Na_2EDTA \cdot 2\ H_2O$. To make the working 1X TB buffer, dilute 10X TB 1:10 in sterile distilled water.
2. 3% Nusieve (FMC, Rockland, Maine, USA), 1% agarose gel in 1X TB.
3. 100 mg/ml ethidium bromide (EtBr; Sigma, St. Louis, MO, USA).
4. Microwave oven.
5. Gel loading dye: 0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol.
6. Molecular weight marker: ΦX174 DNA-Hae III digest (New England Biolabs, Beverly, MA, USA).
7. Electrophoresis gel box and power supply, such as the Minisub™ DNA Cell or Wide Minisub™ Cell and PowerPac 300 (BioRad, Hercules, CA, USA).
8. Designated "post-PCR" micropipettes and aerosol resistant micropipette tips for all post-PCR work (Rainin, Emeryville, CA, USA). These pipettes should be used for any reagent that contains PCR-product or comes into contact with PCR-product; they should never be used in the designated clean area or for PCR setup.

C. Dot Blotting

1. Denaturation solution: 0.4 N NaOH, 25 mM EDTA, 0.01% Orange II dye (Fluka, St. Louis, MO, USA).
2. Biodyne® B nylon membrane (Pall BioSupport Division, Port Washington, NY, USA) cut to fit the dot blot apparatus. Laboratories may wish to invest in silk-screening dot position numbers on the membrane to aid in interpretation. We recommend Palmer Display in San Leandro, CA, USA.
3. Dot blotting apparatus: For automated dot blotting, use the Hydra-96 Microdispenser (Robbins Scientific, Sunnyvale, CA, USA). For manual dot blotting, use either the Convertible™ Filtration Manifold System (BRL Life Technologies, Inc., Gaithersburg, MD, USA) or the Bio-Dot™ apparatus (BioRad, Hercules, CA, USA).
4. Vacuum source.
5. UV Stratalinker (Stratagene, La Jolla, CA, USA).
6. A pair of forceps for handling membranes.
7. A multi-channel pipettor designated for post-PCR work.

D. Hybridization

1. 20X SSPE: 0.11 N NaOH, 3.6 M NaCl, 0.2 M NaH_2PO_4, 0.02 M EDTA, 20% SDS.
2. 17 HRP-labeled DPA1 sequence-specific oligonucleotide probes (SSOPs; Table 2) and 35 HRP-labeled DPB1 SSOPs (Table 3). Oligonucleotide probes can be ordered commercially with HRP covalently linked to the 5′ end. Suggested vendors are Tri-Link (La Jolla, CA, USA), and CyberSyn (Lenni, PA, USA). Probes should be diluted in a solution of 0.5 M NaCl, 50 mM Na_3PO_4, pH 7.5 to a final concentration of 2 μM and stored at 4°C. (Do not freeze.)
3. Seal-A-Meal® bags, 8 in. x 6 in. (Dazey Corp., New Century, KS, USA).
4. Impulse sealer (American International Electric Co., Santa Fe Springs, CA, USA).
5. Glass bowls, such as Pyrex® or Kimax® crystallizing dishes, size 150 mm x 75 mm or 170 mm x 90 mm; watch glasses to cover glass bowls; and vinyl-coated lead weights (VWR, USA).
6. Shaking water baths with plastic bubble covers and temperature control, such as the Hot Shaker (Bellco, Vineland, NJ, USA).
7. A submersible thermometer for each water bath, used to monitor the water bath temperature independently of the machine's own internal temperature controls.
8. Dulbecco's phosphate buffer saline (PBS; 2.68 mM KCl, 137 mM NaCl, 1.47 mM KH_2PO_4, 8 mM Na_2HPO_4, pH 7.4.)
9. Hybridization and stringent wash solutions listed in Tables 2 and 3.

Example of how to make 500 ml of 1X SSPE/0.5% SDS hybridization solution:
Add 25 ml of 20X SSPE to 462.5 ml H_2O. Mix well, add 12.5 ml of 20% SDS, and mix again. Do not add SDS solution directly to SSPE without adding water first or SDS will precipitate out. Once the solution is made, SDS can precipitate out if the room temperature drops below 20°C. To resuspend, heat solution in a 50°C waterbath and mix well.

E. Detection

1. 0.1 M sodium citrate, pH 5.0.
2. 2 mg/ml 3,3′,5,5′-tetramethylbenzidine (TMB; Fluka, St. Louis, MO, USA) in 100% ethanol.
3. 30% hydrogen peroxide (J.T. Baker®, Phillipsburg, NJ, USA).
4. Glass bowl.
5. Rotating platform, such as the Gyrotory® Shaker (New Brunswick Scientific Co., Inc., Edison, NJ, USA).

F. Stripping of Probes for Re-use of Membranes

1. 18% sodium sulfite (Na_2SO_3) stored in an amber bottle.
2. 0.1% SDS solution.
3. Glass bowl.
4. Microwave oven.

G. Interpretation

1. While the results of the DPA1 probe patterns can be interpreted manually, it is strongly recommended that laboratories interested in doing DPB1 typing consult a software engineer about designing a pattern matching program for interpreting the DPB1 probe hit patterns.
2. Polaroid or CCD camera.
3. Probe hit patterns for DPA1 (Table 4) and DPB1 (Table 5).
4. Probe hit scoresheets for DPA1(Table 6) and DPB1 (Table 7).

Procedure

A. Preparation of Genomic DNA

1. Choose any of the recommended kits/methods to obtain genomic DNA for amplification. DNA prepared without the aid of a commercial kit should be resuspended and stored in 1X TE (0.01 M Tris, 0.1 mM EDTA , pH 8).
2. DNA should be prepared in a designated clean area and not come into contact with PCR-product or any reagents, equipment or materials exposed to PCR-product.

B. DP Amplifications

Set up amplifications in a designated clean area.

1. DPA1: Add 50 µl of the DP amplification premix, 1 µl each of primers DPA1-F and DPA1-R (each at 50 µM), 200 ng of control DNA or sample, and sterile water to a final volume of 100 µl. Include at least one no-DNA control (PCR mix with water instead of DNA) in each tray and/or for each amplification premix. Cap the reaction tubes, place in the thermocycler, and start the following amplification program:

soak:	95°C	5 min
35 cycles:	95°C	25 sec
	55°C	45 sec
	72°C	45 sec
soak:	72°C	5 min
hold:	4-10°C	forever

2. DPB1: Add 50 µl of DP amplification premix, 1 µl each of primers UG19 and UG21 (each at 50 µM), 200 ng of control DNA or sample, and sterile distilled water to a final volume of 100 µl. Include at least one no-DNA control in each tray and/or for each amplification premix. Cap the reaction tubes, place in the thermocycler, and start the following amplification program:

soak:	95°C	5 min
35 cycles:	95°C	15 sec
	65°C	1 min
	72°C	15 sec
soak:	72°C	5 min
hold:	4-10°C	forever

Once the amplification is complete, all materials and procedures from this point forward are considered post-PCR.

C. Gel Electrophoresis

1. To determine the efficiency of amplification, examine 3-5 µl of amplicon combined with 1-2 µl of gel loading dye on a 3% Nusieve/1% agarose gel stained with ethidium bromide. (Use approximately 2.5 µl of 100 mg/ml EtBr per 100 ml of agarose, and carefully mix EtBr with melted agarose prior to pouring.)
2. Run at 100 volts until the faster of the two running dyes is at the bottom of the gel. Amplicons of both DPA1 and DPB1 should appear as single, intense bands of just over 300 base pairs and will run approximately the same distance as the fifth fragment of the ΦX174 DNA-*Hae* III molecular weight marker.
3. Amplifications resulting in weak bands on the gel should be repeated.
4. There should be no amplicon in the no-DNA control lane. If a band is present, discard all amplifications and repeat PCR setup with entirely new reagents.

D. Dot Blotting

1. For the DPA1-SSOP assay, it is most convenient to blot the amplicon onto nine membranes, thus enabling two sets of hybridization reactions:
   a. **Automated Dot Blotting:** Using the Hydra Microdispenser, denature remaining amplicon (approximately 90-95 µl) in 100 µl of denaturation solution. Using the Hydra Microdispenser, ensure mixing of amplicon with denaturation solution by dispensing denaturation solution into amplicon, then filling, emptying, and refilling the glass capillary tubes with denatured amplicon. Program the Hydra to dispense 20 µl per dot onto a dry membrane applied to a manifold equipped with 96 holes and attached to a vacuum source. (Be sure to use forceps and wear gloves when handling membranes; do not touch membranes with bare hands.) Repeat blotting 20 µl per dot per membrane until nine membranes are made.

   b. **Manual Dot Blotting:** For nine membranes, add approximately 550 µl of denaturation solution to amplicon using a multi-channel pipettor. Pipette up and down to ensure mixing. Attach the dot blotter to a vacuum and, following the dot blotter manufacturer's protocol, blot 70 µl of denatured amplicon onto membranes pre-wet in distilled water.
2. For the DPB1-SSOP assay, it is most convenient to blot the amplicon onto 14 membranes, thus enabling three sets of hybridizations:
   a. **Automated Dot Blotting:** Using the Hydra Microdispenser, denature remaining amplicon (approximately 90-95 µl) in 200 µl of denaturation solution. Dispense 20 µl onto a dry membrane applied to a manifold equipped with 96 holes and attached to a vacuum source. Repeat blotting 20 µl per dot per membrane until 14 membranes are made.
   b. **Manual Dot Blotting:** Using a multi-channel pipettor, add approximately 900 µl of denaturation solution to amplicon, mix, and blot 70 µl per dot per pre-wet membrane until 14 membranes are made.
3. After DNA has been blotted, immobilize the DNA onto the membrane by UV cross-linking with a Stratalinker at 50 mJ/cm^2.
4. Rinse unbound DNA by boiling the membranes in a glass bowl filled with distilled water or 0.1% SDS for approximately 10 min in a microwave oven.

E. Hybridization

Each individual probe has its own optimal hybridization and wash conditions. Users should pay close attention to the concentration of SSPE used in the hybridization and wash solutions for each probe (Tables 2 and 3). Attention should also be given to the temperatures at which these hybridizations and washes are carried out. A submersible thermometer should be placed in all water baths so that the temperature can be monitored independently of the display on the machine.

1. Place each membrane in a separate plastic Seal-A-Meal® bag and add 10 ml of hybridization solution per 96-sample membrane. (If hybridizing half a membrane, less than 10 ml is adequate; simply use enough solution to cover the membrane.) Add 1 µl of probe (at 2 µM) per ml of hybridization solution and seal each bag with a heat sealer. Ensure that no air is left in the bags when sealing and make the seal as close to the membrane as possible. Submerge the bags containing the membranes in a water bath pre-heated to the desired temperature (Tables 2 & 3) and place lead weights on the corners of the bags to keep them submerged. Do not put the weights directly on top of the membranes, as this can interfere with the hybridization. Set the shaker to approximately 60 rpm and incubate for at least 30 min. To ensure that the water bath remains at the correct temperature, do not remove the lid during the hybridization step.
2. Follow the hybridization step with the indicated stringent wash step (Tables 2 and 3). Remove the membranes from the bags and place them in glass bowls containing stringent wash solution pre-warmed in water baths to the desired wash solution temperatures. Make sure there is enough solution to cover the membranes and allow the membranes to move freely within the bowls when the water bath is shaking. Cover each bowl with a watch glass held in place with a lead weight, close the water bath lid, and set the shaker speed to approximately 60 rpm. Wash for lengths of time and at temperatures indicated in Tables 2 and 3.
3. After the stringent wash step, immediately remove the membranes and place them in a bowl of PBS solution at room temperature with enough liquid to cover all of them. Membranes can be stored in this manner until the detection step.

F. Detection

1. Hybridization of the HRP-labeled probe to the immobilized PCR-product is detected by using the colorless soluble substrate TMB, which is converted to a blue precipitate by HRP in the presence of hydrogen peroxide. In a glass bowl shaking moderately, combine sodium citrate and TMB in a ratio of 20:1. (A total volume of approximately 200 ml will be enough to develop about 10 membranes.) Add hydrogen peroxide to a final concentration of 0.0015%, then add two or three membranes at a time. As soon as a blue precipitate appears, transfer membranes to a glass bowl shaking moderately and containing enough water to cover all membranes. This will stop the color development. Shake for five minutes and replace water. It is best to develop only a few membranes at a time in order to prevent over-development. The best indicator of the proper time to remove the membranes from the development solution is the hybridization patterns of the control samples. As soon as the controls turn blue for the correct probe, remove the membranes. They are fully developed when the dots with the appropriate positive control DNAs are blue and the dots with the negative control DNAs are still white.
2. Immediately record results by photographing each membrane with a Polaroid or CCD camera. Do not allow the membranes to sit for very long after development, as the background signal from non-specific hybridization may increase, making it difficult to interpret the results.
3. Record probe hit patterns for each sample using the probe hit scoresheets in Tables 6 (DPA1) and 7 (DPB1).

G. Stripping of Probes for Re-use of Membranes

1. The DNA immobilized on the membranes is stable, so the membranes can be repeatedly reused for subsequent hybridizations after the bound probe is removed. It is best to strip the probes from the membranes as soon as the results have been recorded. If the membranes are left for longer than a few hours, the blue precipitate becomes difficult to remove and the membranes may become discolored.

2. First, to remove the blue precipitate, submerge the membranes in a bowl of warm distilled water (approximately 750 ml for 10 membranes) mixed with 5-10 ml of 18% Na_2SO_3. Shake until the blue color disappears.
3. Rinse the membranes thoroughly in distilled water to remove the Na_2SO_3.
4. Second, to remove the probe, submerge membranes completely in a 0.1% SDS solution (approximately 750 ml per 10 membranes) and heat to boiling (10-15 min) in a microwave oven.
5. Rinse the membranes in distilled water. They are now ready to be used with the next set of probes. When the assay is complete, the membranes can be air-dried and stored in Seal-a-Meal bags.

H. Interpretation

1. Interpretation of DPA1 hybridization results can be performed by recording the probe hybridization patterns on a scoresheet like the one shown in Table 6. Interpretation can be done manually using the probe reactivity patterns provided at the top of the scoresheet. With the present number of 11 DPA1 alleles, one ambiguous genotype may arise when interpreting probe hybridization results; the DPA1*0103,*02022 genotype cannot be distinguished from DPA1*02013,*0302. The group-specific primer AB139 (Table 1) should be used to selectively amplify either the *0103 or *02013 allele. The resulting amplicon can then be typed with the original 17 SSOPs, and the phase of the sequence motifs detected by the SSOPs can be determined, resolving the ambiguity.
2. Because there are over 75 DPB1 alleles, and because a large majority of the alleles result from shuffling of the same sequence motifs in the six regions of variability, manual interpretation of DPB1 hybridization results becomes quite tedious and difficult. Consequently, interpretation software is highly recommended. Depending on the population being typed, one may obtain a high percentage of ambiguous types in which the phase of the sequence motifs (as indicated by positive probes) cannot be determined. These ambiguities can be resolved by doing a second amplification using a group-specific primer to selectively amplify one of the two alleles in a heterozygous sample (Table 1). The resulting amplicon should then be typed with a subset of the original probes, establishing the phase of the sequence motifs. Table 8 outlines the most common ambiguities uncovered in our analyses of over 3,500 samples and the sequence-specific primers and probes used to resolve them. As the ambiguous genotypes will vary between populations, the user may have to design additional group-specific primers.

Procedure Notes

Below are possible problems one may encounter in performing the DPA1 and DPB1 typing assays. For each possible problem, one or more solutions are presented.

1. Complete PCR dropout (no PCR amplification of any sample):
   The PCR reaction mix may have been prepared incorrectly, or the wrong amplification program may have been used. Repeat the amplification with new reagents and check that the correct amplification profile is used.
2. Sporadic PCR dropouts or weak amplifications:
   a. The sample may have contained an insufficient amount of DNA; add more sample to amplification.
   b. An inhibitor may have been present in the DNA sample; the two most common inhibitory problems are heme carried over from the sample preparation or too much EDTA in the solution used to resuspend the genomic DNA. Repeat sample extraction, removing all heme. Check the concentration of EDTA in the buffer used to resuspend DNA; it should be less than 1 mM.
   c. DNA may not have been added; repeat amplification.
3. Positive band in the no-DNA control lane after gel electrophoresis:
   Reaction mix may be contaminated with PCR-product or genomic DNA. Discard amplification and repeat with entirely new reagents.
4. Probe signal is positive on negative controls:
   a. Cross-hybridization may have occurred because the stringency of the wash step was too low (the temperature in the water bath was too low or the salt concentration in the hybridization or wash solutions was too high). Check the water bath temperature using the submersible thermometer. If the temperature was accurate, prepare new hybridization and wash solutions.
   b. The membrane may have been left too long in development solution; strip probe from the membrane, then repeat hybridization, wash, and development steps, removing the membrane as soon as the positive control dots begin to turn blue.
5. No probe signal present:
   a. Probe may not have been added; repeat hybridization, wash, and development steps.
   b. Stringency may have been too high. Check water bath temperature to see if it was too high. If the temperature was accurate, prepare new hybridization and wash solutions.
   c. Probe may have stopped working (HRP inactivated); strip probe from the membrane, repeat hybridization, wash, and development steps, and if no results are obtained, re-order the probe.
6. Weak probe signals on certain samples:
   a. An insufficient amount of DNA may have been blotted on the membrane; compare the control probe (DPA1: 40-TVWHLE; DPB1: 37-RFDSDV) intensity on the weak sample with that of the other positive samples. If the questionable sample has a weak control probe signal as well, it is probably positive for the faint probe; however, it is recommended that the sample be re-amplified and typed again.

b. The probe may not have hybridized equally well to all of the samples on the membrane. Repeat hybridization, making sure that the membrane is completely covered by the hybridization solution and that the bag is completely submerged in the water bath.
c. The sample may contain a mutant sequence in the region complementary to the probe, preventing efficient hybridization. If the same results are obtained a second time, consider cloning and sequencing the sample to confirm the sequence.

7. Sample has a unique probe hybridization pattern. Make sure probes display the hybridization patterns consistent with the expected patterns for the positive controls. If they are correct, the sample may contain a new allele. If the same results are obtained for the sample a second time, clone and sequence it to confirm the sequence.
8. Finally, as the sequences of new alleles are reported in the literature, additional ambiguities in the interpretation of the results might be introduced. Additional probes and group-specific primers may have to be designed to resolve these ambiguities. However, the decision to add additional reagents to an assay should depend on the frequency of the new allele in the population the user is studying, as well as on the level of resolution the user wishes to achieve. Although the sequences of new DP alleles are constantly being reported, most appear to be extremely infrequent. For example, as the authors were completing this chapter, the sequence of the DPA1*0203 allele was uncovered.[24] The probe hybridization pattern of this allele, which was found in a single Brazilian individual, introduces a new ambiguity in the DPA1-typing system described here. The heterozygous genotypes DPA1*02011,02013 and DPA1*02013,0203 cannot be distinguished. This ambiguity could be resolved by introducing a new probe for the methionine residue at position 31 in the DPA1 molecule; however, the frequency of both the DPA1*02013 and 0203 alleles is so low (DPA1*02013 was found in a single individual in the Cameroon[10]) that it is very unlikely that this genotype will appear in any population study.

Acknowledgments

This work was supported in part by NIH grant AI29042. We are grateful to S. Mack and H. Erlich for valuable comments on this manuscript.

References

1. Termijtelen, A., Naipal-van den Berge, S., Suwandi-Thung, L. and van Rood, J.J. (1984) The influence of matching for SB on MLC typing is significant but marginal. *Scand. J. Immunol.* 19, 265-268.
2. Saiki, R.K., Scharf, S., Faloona, F., Mullis, K.B., Horn, G.T., Erlich, H.A. and Arnheim, N. (1985) Enzymatic amplification of b-globin genome sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science* 230, 1350-1354.
3. Wank, R., Schendel, D.J., Hansen, J.A. and Dupont, B. (1978) The lymphocyte restimulation system: evaluation by intra-HLA-D group priming. *Immunogenetics* 6, 107-115.
4. Mawas, C., Charmot, D. and Mercier, P. (1980) Split of HLA-D into two regions alpha and beta by a recombination between HLA-D and GLO. I. Study in a family and primed lymphocyte typing for determinants coded by the beta region. *Tissue Antigens* 15, 458-466.
5. Shaw, S., Johnson, A.H. and Shearer, G.M. (1980) Evidence for a new segregant series of B cell antigens that are encoded in the HLA-D region and that stimulate secondary allogeneic proliferative and cytotoxic responses. *J. Exp. Med.* 152, 565-580.
6. Odum, N., Hofmann B., Hyldig-Nielsen, J.J., Jakobsen, B.K., Morling N., Platz, P., Ryder, L.P. and Svejgaard, A. (1987) A new supertypic HLA-DP related determinant detected by primed lymphocyte typing. *Tissue Antigens* 29, 101-109.
7. Lotteau, V., Teyton, L., Tongio, M.-M., Soulier, A., Thomsen, M., Sasportes, M. and Charron, D. (1987). Biochemical polymorphism of the HLA-DP heavy chain. *Immunogenetics* 25, 403-407.
8. Hyldig-Neilsen, J.J., Morling, N., Odum, N.H., Ryder, L.P., Platz, P., Jakobsen, B.K. and Svejgaard, A. (1987) Restriction fragment length polymorphism of the HLA-DP subregion and correlations to HLA-DP phenotypes. *Proc. Natl. Acad. Sci. USA* 84, 1644-1648.
9. Bodmer, J.G., Marsh, S.G.E., Albert, E.D., Bodmer, W.F., Bontrop, R.E., Charron, D., Dupont, B., Erlich, H.A., Fauchet, R., Mach, B., Mayr, W.R.,Parham, P., Sasazuki, T., Schreuder, G.M.Th., Strominger, J.L., Svejgaard, A. and Terasaki, P.I. (1997) Nomenclature for factors of the HLA system, 1996. *Tissue Antigens* 49, 297-321.
10. Steiner, L.L., Cavalli, A., Zimmerman, P.A., Boatin, B.A., Titanji, V.P.K., Bradley, J.E., Lucius, R., Nutman, T.B. and Begovich, A.B. (1998) Three new DP alleles identified in sub-Saharan Africa: DPB1*7401, DPA1*02013, and DPA1*0302 (submitted).
11. Tsuji, K., Aizawa, M. and Sasazuki, T., eds. (1992) HLA 1991: *Proceedings of the Eleventh International Workshop and Conference.* Oxford Scientific Publications, Oxford, England.
12. Terasaki, P.I. and Gjertson, D.W., eds. (1997) HLA 1997. UCLA Tissue Typing Laboratory, Los Angeles, CA.
13. Charron, D., ed. (1997) *Genetic Diversity of HLA: Functional and Medical Implications.* EDK, Paris, France. .
14. Hoffman, R.W., Shaw, S., Francis, L.C., Larsen, M.G., Petersen, R.A., Chylack, L.T. and Glass, D.N. (1986) HLA-DP antigens in patients with pauciarticular juvenile rheumatoid arthritis. *Arthritis Rheum.* 29, 1057-1062.
15. Odum, N., Morling, N., Friis, J., Heilmann, C., Hyldig-Nielsen, J.J., Jakobsen, B.K., Pedersen, F.K., Platz, P., Ryder, L.P. and Svejgaard, A. (1986) Increased frequency of HLA-DPw2 in pauciarticular onset juvenile chronic arthritis. *Tissue Antigens* 28, 245-250.
16. Begovich, A.B., Bugawan, T.L., Nepom, B.S., Klitz, W., Nepom, G.T. and Erlich, H.A. (1989) A specific HLA-DPB allele is associated with pauciarticular juvenile rheumatoid arthritis but not adult rheumatoid arthritis. *Proc. Natl. Acad. Sci.* 86, 9489-9493.
17. Fernandez-Vina, M.A., Fink, C.W. and Stasney, P. (1990) HLA antigens in juvenile arthritis. Pauciarticular and polyarticular juvenile arthritis are immunogenetically distinct. *Arthritis Rheum.* 33, 1787-1797.

18. Erlich, H.A., Rotter, J.I., Chang, J.D., Shaw, S.J., Raffel, L.J., Klitz, W., Bugawan, T.L. and Zeidler, A. (1996) Association of HLA-DPB1*0301 with IDDM in Mexican-Americans. *Diabetes* 45, 610-614.
19. Noble, J.A., Valdes, A.M., Cook, M., Klitz, W., Thomson, G. and Erlich, H.A. (1996) The role of HLA class II genes in insulin-dependent diabetes mellitus: Molecular analysis of 180 Caucasian, multiplex families. *Am. J. Hum. Genet.* 59, 1134-1148.
20. Richeldi, L., Sorrentino, R. and Saltini, C. (1993) HLA-DPB1 glutamate 69: A genetic marker of beryllium disease. *Science* 262, 242-244.
21. Newman, L.S. (1993) To Be^{2+} or not to Be^{2+}: Immunogenetics and occupational exposure. *Science* 262, 197-198.
22. Maniatis, T., Fritsch, E.F. and Sambrook, J., eds. (1982) *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
23. Bugawan, T.L., Begovich, A.B. and Erlich, H.A. (1990) Rapid HLA-DPB typing using enzymatically amplified DNA and nonradioactive sequence-specific oligonucleotide probes. *Immunogenetics* 32, 231-241.
24. Muntau, B., Thye, T., Pirmez, C. and Horstmann, R.D. (1997) A novel DPA1 allele (DPA1*0203) composed of known epitopes. *Tissue Antigens* 49, 668-669.

Table 1. DP Group-Specific Primers

Primer Name	Locus	Sequence Variant[a]	Primer Sequence	Alleles Amplified[b]	Amplification Profile
AB139[c]	DPA1	A@11(F)	GGATCCATGTGTCAACTTATGCCGC	0103, 02013	95°C-20 sec/60°C -1 min/72°C -20 sec
PM012[d]	DPB1	L@11(F)	AGAGAATTACGTGTACCAGTT	2101, 3601	95°C-15 sec/65°C -1 min/72°C -15 sec
PM023[e]	DPB1	A@55(R)	GTCCTTCTGGCTGTTCCAGTACTCCGCAG	01011, 0401, 26012, 5501	95°C-30 sec/65°C -1 min/72°C -30 sec
PM024[e]	DPB1	D@55(R)	GTCCTTCTGGCTGTTCCAGTACTCCTCAT	02012, 0402, 4801, 4901, 5101	95°C-15 sec/70°C -1 min/72°C -15 sec
AB1002[d]	DPB1	E@56(F)	GAGCTGGGGCGGCCTGATGA	02012, 0402	95°C-30 sec/65°C -1 min/72°C -30 sec
PM026[e]	DPB1	L@65(R)	TGCCCGCTTCTCCTCCAGGAG	0301, 1401, 2501, 4501	95°C-15 sec/70°C -1 min/72°C -15 sec
PM016[e]	DPB1	V@76(R)	CTCGTAGTTGTGTCTGCATAC	01011, 0301, 0801, 0901, 1001, 1401, 2501, 2901, 3501, 4501, 5001	95°C-15 sec/65°C -1 min/72°C -15 sec

[a] Indicates amino acid distinguished at the 3′ end of the primer and the location of the amino acid in the protein alignment. (F) indicates forward primer; (R) indicates reverse primer.
[b] Alleles listed are those present in common ambiguous genotypes that need to be resolved; it is not a complete list of all alleles that will be amplified by each primer.
[c] Used in combination with the 3′ primer DPA1-R.
[d] Used in combination with the 3′ primer UG21.
[e] Used in combination with the 5′ primer UG1

Table 2. DPA1 SSOPs: Sequences and Hybridization Conditions

Probe Name	Sequence Variant[a]	HRP-SSOP Sequence 5′ to 3′[b]	Hybridization[c]	Wash[d]
PVM1	8-YAAF	CTTATGCCGCGTTTGTAC	5x	1x
PVM3	8-YAMF	CTTATGCCATGTTTGTAC	1x	1x
PVM27	13-VQTH1	GTACAGACGCATAGA	1x	0.4x
PVM28	12-VQTH2	TTTGTACAGACCCATAGA	1x	0.4x (15min)
PVM5	28-EDEQ	AGATGAGCAGTTCTATGT	1x	1x (15min)
PVM22	25-DDEM1	ATTTGATGACGATGAGAT	5x	0.4x
PVM30	25-DDEM2	TCTCATCTTCATCAAAT	3x	1x
AB135	36-DKK1	CTGGATAAAAAGGAGAC	5x	1x
AB130	36-DKK2	TCCTTCTTGTCCAGAT	3x	1x
PVM31	36-DKK3	GTCTCCTTCTTATCCAG	1x	1x
PVM24	41-TVWHLE	ACCGTCTGGCATCTGAG	1x	0.1x (15 min)
PVM6	47-FGQA	AGTTTGGCCAAGCCTTTT	2x	0.2x
PVM9	63-AISN	TTGCTATATCGAACAACA	5x	1x (15 min)
AB138	63-AILN	TGTTGTTCAATATAGCAA	5x	1x
PVM11	70-IAIQ	TTGAATATCGCTATCCAG	5x	1x
LS004	81-QATN	CAGGCCACCAACGGTACG	5x	0.4x (15 min)
LS005	81-QAAN	CGTACCATTGGCGGCCTG	5x	0.4x (15 min)

[a] Indicates the polymorphic sequence motif detected by the probe and the 5′ residue at which the probe starts.
[b] Probes are labeled at the 5′ end with horseradish peroxidase.
[c] All hybridization solutions contain 0.5% SDS in addition to SSPE. Table indicates the SSPE concentration used for each probe. [20X SSPE: 0.11 NaOH; 3.6 M NaCl; 0.2 M NaH_2PO_4; 0.02 M EDTA]. All hybridizations are done in a 42°C waterbath for 30 min.
[d] All wash solutions contain 0.1% SDS in addition to SSPE. Table indicates the SSPE concentration used for each probe. All washes are done in a 42°C water bath for 12 min unless otherwise indicated.

Table 3. DPB1 SSOPs: Sequences and Hybridization Conditions

Probe Name	Sequence Variant[a]	HRP-SSOP Sequence 5′ to 3′[b]	Hybridization (°C)[c]	Wash (°C)[d]
DPB96	5-LFQG	GAATTACCTTTTCCGGGGACG	4X/50°	1X/50°
AB127	6-VYQL	CGTAACTGGTACACGTAA	4X/50°	1X/50°
DPB4	6-VHQL	TTACGTGCACCAGTTACG	4X/50°	1X/50°
DPB7	7-VYQG	CGTCCCTGGTACACGTA	4X/50°	1X/50°
AB117	33-EEFARF	AGGAGTTCGCGCGCTT	5X/55°	0.1X/50°
DPB11	33-EEFVRF	AGCGCACGAACTCCT	4X/50°	1X/50°
DPB12	33-EELVRF	AGGAGCTCGTGCGCTT	4X/50°	1X/50°
DPB18	31-QEYARF	AACCGGCAGGAGTACG	4X/50°	1X/50°
DPB8	32-EEYARF	GGGAGGAGTACGCG	4X/50°	1X/50°
DPB19	54-AAE	CCTGCTGCGGAGTACT	4X/50°	1X/50°
DB101	53-DEE	CCAGTACTCCTCATCAGGC	1X/50°	0.1X/42° (12 min)
DPB27	54-EAE	CCTGAGGCGGAGTACT	4X/50°	1X/50°
DPB92	55-DED	GTTCCAGTAGTCCTCATC	4X/50°	1X/50°
AB112	54-DEV	CCTGATGAGGTGTACTG	3X/50°	0.2X/42° (12 min)
DB34	64-ILEEK	GACATCCTGGAGGAGAAGC	2X/55°	0.1X/42°
DPB127	64-ILEEE	CTCCTCCTCCAGGATGTC	4X/50°	1X/50°
DPB104	64-LLEEK	GACCTCCTGGGGGAGAAGC	4X/50°	1X/50°
DB62	64-LLEEE	GACCTCCTGGAGGAGGAG	3X/50°	0.1X/42°
DB63	64-LLEER	GACCTCCTGGAGGAGAGG	2X/55°	0.1X/42° (12 min)
PVM32	62-FLEEE	CCTCCAGGAAGTCCTTCT	1X/42°	0.2X/42°(12 min)
DPB109	64-LL*EK	ACCTCCTGTAGGAGAAG	4X/42°	1X/42°
DPB110	63-NLEEK	AAGGACAACCTGGAG	4X/42°	1X/42°
AB96	73-M	TGTCTGCACATCCTGTCCG	1X/42°	0.2X/50°
AB97	73-V	TGTCTGCATACCCTGTCCG	2X/42°	0.2X/50°
AB98	73-I	CGGACAGGATATGCAGACA	2X/42°	0.4X/50°
DPB65	82-GGPM	GGGCCCGCCCAGCTC	4X/50°	1X/50°
AB123	81-VGPM	CGAGCTGGTCGGGCCCA	3X/55°	0.1X/55° (10 min)
DB77	83-DEAV	CTGGACGAGGCCGTG	3X/50°	0.1X/42° (12 min)
PVM16	14-ECYPFNG	CCATTAAACGGGTAGCAT	1X/42°	0.4X/42° (12 min)
PVM17	14-ECYAFNG	ATGCTACGCGTTTAATGG	5X/42°	0.1X/42° (12 min)
PVM12	41-DVGEFR2	GACGTGGGAGAGTTCCGG	2X/50°	0.1X/50° (12 min)
PVM13	41-DVGEFR1	CCGGAACTCCCCCACGTC	2X/50°	0.1X/50° (12 min)
PVM20	29-IYNREE2	CTCCTGCCTGTTGTAGA	5X/42°	0.4X/42° (12 min)
PVM21	29-IYNREE1	TCTACAACCGGCAGGAG	5X/42°	0.1X/42° (12 min)
DPB131	37-RFDSDV	GCTTCGACAGCGACGT	4X/50°	1X/50°

[a] Indicates the polymorphic sequence motif detected by the probe and the 5′ residue at which the probe starts.
[b] Probes are labeled at the 5′ end with horseradish peroxidase.
[c] All hybridization solutions contain 0.5% SDS in addition to SSPE. Table indicates the SSPE concentration used for each probe. [20X SSPE: 0.11 NaOH; 3.6 M NaCl; 0.2 M NaH_2PO_4; 0.02 M EDTA]. All hybridizations are done for 30 min.
[d] All wash solutions contain 0.1% SDS in addition to SSPE. Table indicates the SSPE concentration used for each probe. All washes are 15 min unless otherwise indicated.

Table 4. DPA1 Probe Hit Patterns

Probe	DPA1 Allele										
	103	**104**	**105**	**2011**	**2012**	**2013**	**2021**	**2022**	**301**	**302**	**401**
8-YAAF	+	+	+	+	+	+					+
8-YAMF							+	+	+	+	
13-VQTH1	+	+	+		+	+					+
12-VQTH2				+			+	+	+	+	
28-EDEQ				+	+	+	+	+			
25-DDEM1	+										
25-DDEM2	+		+	+	+	+	+	+	+	+	
36-DKK1				+	+						
36-DKK2	+	+	+			+		+	+	+	+
36-DKK3							+				
47-FGQA	+	+	+						+	+	
63-AISN									+		
63-AILN	+	+	+	+	+	+	+	+		+	+
70-IAIQ											+
81-QATN	+	+							+	+	
81-QAAN			+	+	+	+	+	+			+
40-TVWHLE	+	+	+	+	+	+	+	+	+	+	+

Table 5. DPB1 Probe Hit Patterns

| Probe | DPB1 Allele | | |

Table 5. DPB1 Probe Hit Patterns (continued)

Probe	3701	3801	3901	4001	4101	4401	4501	4601	4701	4801	4901	5001	5101	5201	5301	5401	5501	5601	5701	5801	5901	6001	6101N	6201	6301	6401N	6501	6601	6701	6801	6901	7001a	7101	7201	7301	7401
5-LFQG		+	+	+	+			+	+	+	+		+		+				+		+	+		+	+		+			+			+	+	+	
6-VYQL	+					+								+				+					+								+	+				+
6-VHQL							+									+	+			+									+	+						
7-VYQG												+																								
33-EEFARF													+					+											+					+		
33-EEFVRF	+				+		+	+	+			+		+		+	+		+		+	+	+			+			+	+	+	+	+		+	
33-EELVRF		+				+				+										+				+	+											
31-QEYARF																																				+
32-EEYARF			+	+							+				+												+									
54-AAE			+	+										+			+	+		+				+	+		+	+	+				+	+		+
53-DEE	+				+		+			+	+		+		+							+	+							+					+	
54-EAE		+							+							+																				
55-DED						+		+				+							+				+			+						+	+				
54-DEV																																					
64-ILEEK		+	+	+							+		+		+									+	+		+									
64-ILEEE	+							+	+	+						+	+			+														+			
64-LLEEK							+					+		+				+	+		+		f							+			+		+	+	
64-LLEEE						+																				+											
64-LLEER																																+					+
62-FLEEE					+																																
63-NLEEK																						+															
64-LL*EK																							+														
73-M		+	+	+	+			+	+	+	+		+		+		+			+	+	+		+	+	+		+			+		+	+		+
73-V	+					+	+					+		+		+		+	+				+				+		+	+		+			+	
73-I																																					
82-GGPM			+		+			+	+	+	+		+								+	+						+					+	+	+	
81-VGPM				+											+									+													+
83-DEAV	+	+				+	+					+		+		+	+	+	+	+			+		+	+	+		?	+	+	+				
41-DVGEFR1																																					+
41-DVGEFR2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
14-ECYPFNG		+																																			
14-ECYAFNG	+		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
29-IYNREE1																																					
29-IYNREE2																																					+
37-RFDSDV	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

[a]DPB1*0301 and the newly discovered *7001 allele have identical probe hybridization patterns. They differ by a single nucleotide in codon 9 that results in a predicted amino acid change (Y to D); however, this single nucleotide does not destabilize the hybridization of the probe for the VYQL motif (AB127) with the VDQL sequence motif. To resolve these two alleles a new probe capable of detecting the nucleotide difference between these two alleles is under development.

[b]The probe hybridization patterns for alleles *20011 and *20012 are identical; they differ by a single nucleotide at position 3 in codon 91.

?: There is a single nucleotide difference between the probe and the target sequence. DNA was unavailable, so specificity of the probe is unknown.

f (faint): A single nucleotide difference between the probe and target sequence destabilizes the probe binding and decreases the intensity of the signal.

Table 6. DPA1 Probe Hit Scoresheet

		0103, 0104, 0105, 02011/2/3, 0401	02021/2, 0301, 0302	0103, 0104, 0105, 02012/3, 0401	02011, 02021/2, 0301, 0302	02011/2/3, 02021/2	0104	All but 0104 & 0401	02011, 02012	All but 02011/2 & 02021	02021	All	0103, 0104, 0105, 0301, 0302	0301	All but 0301	0401	0103, 0104, 0301, 0302,	0105, 02011/2/3, 02021/2 0401
Sample[a]	**DPA1 Type**	**YAAF**	**YAMF**	**VQTH1**	**VQTH2**	**EDEQ**	**DDEM1**	**DDEM2**	**DKK1**	**DKK2**	**DKK3**	**ALL**	**FGQA**	**AISN**	**AILN**	**IAIQ**	**QATN**	**QAAN**
1 LBUF	2011	+			+	+		+	+			+			+			+
2 CB6B	2021		+		+	+		+			+	+			+			+
3 AMAI	301		+		+			+		+		+	+	+			+	
4 T7526	401	+		+						+		+			+	+		+
5 SK	104	+		+			+			+		+	+		+		+	
6																		
7																		
8																		
9																		
10																		
11																		
12																		

[a]Samples LBUF, CB6B, AMAI, T7526 and SK are the control DNA panel.

Table 7. DPB1 Probe Hit Scoresheet

Sample[a]	DPB1 Type	LFQG	VYQL	VHQL	VYQG	EEFARF	EEFVRF	EELVRF	QEYARF	EEYARF	AAE	DEE	EAE	DED	DEV	ILEEK	ILEEE	LLEEK	LLEEE	LLEER	FLEEE	NLEEK	LL*EK	M	V	I	GGPM	VGPM	DEAV	DVGEFR1	DVGEFR2	ECYPFNG	ECYAFNG	IYNREE1	IYNRRE2	RFDSDV
1 LKT3	501	+						+					+			+								+					+		+		+			+
2 LBUF	1701			+			+							+			+							+					+		+		+			+
3 TER81	0101, 1301		+		+					+	+					+	+								+	+			+		+		+			+
4 JY	0201, 0401	+				+	+				+	+				+	+							+			+				+		+			+
5 BIN40	0301, 0601		+				+							+				+	+					+	+				+		+		+			+
6 PLH	1501				+				+		+									+				+				+		+			+		+	+
7 CRK	01011, 11011		+		+				+	+	+					+				+				+	+				+	+	+		+		+	+
8 AH696	11012		+						+		+									+				+					+	+			+	+		+
9 NG78	3201	+					+								+		+							+			+				+		+			+
10 SE53	3801	+						+					+			+								+					+		+	+				+
11 T93	4101	+					+					+									+			+			+				+		+			+
12 C23	6001	+					+					+										+		+			+				+		+			+
1 C53	6101N		+				+							+				f					+		+				+		+		+			+
2																																				
3																																				
4																																				
5																																				
6																																				
7																																				
8																																				
9																																				
10																																				
11																																				
12																																				

[a]Samples LKT3, LBUF, TER81, JY, BIN40, PLH, CRK, AH696, NG78, SE53, T93, C23, and C53 are the control DNA panel.
f (faint): A single nucleotide difference between the probe and target sequence destabilizes the probe binding and decreases the intensity of the signal.

Table 8. Common Ambiguous DPB1 Genotypes

Ambiguous Genotypes	Sequence-Specific Primer	Alleles Amplified	Probes to Resolve Genotype
01011, 0301 ↔ 26012, 5001	A@55(R)	01011, 26012	VYQL, VYQG, EEFVRF, EEYARF, AAE, DED
01011, 2001 ↔ 2701, 5001	V@76(R)	01011, 5001	VYQL, VYQG, EEFVRF, EEYARF, AAE, DED, IK, LK, M, V
0201, 0202 ↔ 4701, 4801	D@55(R)	0201, 4801	LFQG, EEFVRF, EELVRF, DEE, EAE
0201, 0301 ↔ 2501, 4601	V@76(R)	0301, 2501	LFQG, VYQL, EEFVRF, DEE, DED, IE, LK, M, V
0201, 0401 ↔ 0402, 3301 ↔ 5101, 7101[a]	E@56(F)	0201, 0402, 5101	AAE, DEE, IK, IE, M, GGPM
0201, 0501 ↔ 0402, 2201	E@56(F)	0201, 0402	DEE, EAE, IK, IE, M, GGPM, DEAV
0201, 0901 ↔ 1001, 4601	V@76(R)	0901, 1001	LFQG, VHQL, EEFVRF, DEE, DED, IE, M, V
0201, 1401 ↔ 4501, 4601 ↔ 1701, 7301	V@76(R)	1401, 4501, 7301	LFQG, VHQL, EEFVRF, DEE, DED, IE, LK, M, V
0201, 3501 ↔ 0402, 0901	V@76(R)	0901, 3501	LFQG, VHQL, EEFVRF, DEE, DED, IK, IE, M, V
0301, 0601 ↔ 2001, 2901 ↔ 0301, 6401N[b]	V@76(R)	0301, 2901	VYQL, EEFVRF, DED, LK, LE, M, V
0301, 1001 ↔ 0901, 2501 ↔ 1401,3701	L@65(R)	0301, 1401, 2501	VYQL, VHQL, EEFVRF, DEE, DED, LK, IE
0301, 1601 ↔ 0801, 2001	V@76(R)	0301, 0801	LFQG, VYQL, EEFVRF, DEE, DED, IE, LK, M, V
0301, 1701 ↔ 0901, 2001	V@76(R)	0301, 0901	VYQL, VHQL, EEFVRF, DED, IE, LK, M, V
0401, 0402 ↔ 2301, 5101	D@55(R)	0402, 5101	LFQG, EEFARF, EEFVRF, AAE, DEE
0401, 0901 ↔ 3301, 3501	V@76(R)	0901, 3501	LFQG, VHQL, EEFARF, EEFVRF, AAE, DED, IK, IE M, V,
0401, 3001 ↔ 2401, 5501	A@55(R)	0401, 5501	LFQG, VHQL, EEFARF, EEFVRF, AAE, EAE
0402, 3901 ↔ 2301, 4901	D@55(R)	0402, 4901	LFQG, EEFVRF, EEYARF, AAE, DEE
0501, 0901 ↔ 2201, 3501	V@76(R)	0901, 3501	LFQG, VHQL, EEFVRF, EELVRF, EAE, DED, IK, IE, M, V
0501, 2101 ↔ 2201, 3601	L@11(F)	2101, 3601	LFQG, VYQL, EELVRF, EAE, IK, IE, M, DEAV
0901, 3601 ↔ 2101, 3501	V@76(R)	0901, 3501	VYQL, VHQL, EEFVRF, EELVRF, EAE, DED, IK, IE, M, V
0901, 4501 ↔ 1001, 1401	L@65(R)	1401, 4501	VHQL, EEFVRF, DEE, DED, IE, LK

[a]With the recent discovery of the DPB1*7101 allele, the *5101,*7101 genotype was introduced into this ambiguous combination. Using the sequence-specific primer E@56, the *0402 and *5101 alleles have the same hybridization pattern; consequently the *0402,*3301 and *5101,*7101 genotypes cannot be distinguished. However, the DPB1*3301, *5101, and *7101 alleles are so rare that all samples with this ambiguous probe hybridization pattern have been shown to be *0201,*0401.

[b]With the recent report of the DPB1*6401N allele, the *0301,*6401N genotype was introduced into this ambiguous combination. Using the sequence-specific primer V@76 the *0301,*0601 and *0301,*6401N genotypes cannot be resolved; however, this ambiguity can be resolved by introducing a probe for the stop codon found at position 7 in the rare *6401N allele.

Analysis of HLA Class II DRB1 Alleles Using PCR-RFLP

Julio C. Delgado, Doreen E. Sese, Edgar L. Milford, and Edmond J. Yunis

Purpose

Molecular variants of Class II genes were detected using characteristic variation in the length of restriction endonuclease digests of genomic DNA as early as 1982.[1] This early form of Restriction Fragment Length Polymorphism (RFLP) analysis required the use of radiolabeled probes to identify electrophoretic fragments which could be attributed to Class II genes. Though useful, the method was plagued by the high background from irrelevant genomic DNA fragments and the difficulty of finding probes which were at once inclusive of all alleles at a locus and also locus-specific. The application of the Polymerase Chain Reaction (PCR)[2] to RFLP Class II typing made it possible to selectively amplify allele(s) at a single locus, thereby eliminating these background and specificity problems. The polymerase chain reaction also allowed for the development of several other Class II typing methodologies: PCR-SSOP, PCR-SSP, and PCR-SBT.[3-6] Although other Class II loci (DRB3, DRB4, DRB5, DQA, and DQB) are amenable to typing by PCR-RFLP,[7-9] this chapter will focus on the DRB1 locus.

Polymorphic motifs of the DRB1 gene which are characteristic of previously sequenced alleles can be detected using PCR-RFLP. Group specific amplifications of the DRB1 second exon are performed. The resulting amplicons are then incubated with selected restriction endonucleases which recognize specific 4-8 base pair sequences of double stranded DNA and cut it to produce fragments of predicted size for a given set of alleles. These fragments are electrophoretically separated and visualized on an agarose gel. Depending on the number of primers and enzymes selected, this method can be used to obtain results at serologic through allele level resolution (Table 1).[10] PCR-RFLP works well for low (<10) to medium (<75) numbers of samples per week, and is an economic alternative which can be used alone or in combination with serology and other DNA methods. It can be implemented with minimal capital expenditure and the interpretation does not require a qualitative subjective decision about positivity or negativity of probe signals, but rather depends on the absolute presence or absence of electrophoretic bands. Turn around time varies from 6 hours to 3 days depending on sample volume, resolution required, technologists, and equipment.

Specimens

Purified genomic DNA may be extracted from any source of nucleated cells: peripheral blood, lymph node, spleen, buccal scrapings, etc. Simple lysate preparations are not recommended because intracellular proteins may interfere with the amplifications. Please refer to the DNA Extraction Methods chapter for complete information about purifying DNA. The quality and quantity of each sample should be spectrophotometrically determined. Samples with less than 500ng DNA or greater than 60% protein contamination are not acceptable for PCR-RFLP. Samples may be stored "short term" (<6 months) at 4°C and "long term" (indefinitely) at -20°C.

Protocols

A. DRB1 Group Specific Amplications

Each sample is tested with six different sets of primers which amplify different groups of alleles. Each sample should amplify with at least one set of primers, but no more than two sets of primers.

DRB1 Primer Set	Serological Equivalent or Alleles Amplified	Amplicon Size
G1+B	DR1	261bp
G2+B	DR2	261bp
G3+B	DR3,5,6,8	266bp
G4+B	DR4,*1122,*1410	263bp
G5F+G5R	DR7,9	199bp
G6F+G6R	DRB1*1001	225bp

DRB1 Group Specific Primer Sequences		(Length)
G1: (Sense)*	5′- TTC TTg Tgg CAg CTT AAg TT -3′	(20mer)
G2: (Sense)*	5′- TTC CTg Tgg CAg CCT AAg Agg -3′	(21mer)
G3: (Sense)*	5′- CAC gTT TCT Tgg AgT ACT CTA C -3′	(22mer)
G4: (Sense)*	5′- gTT TCT Tgg AgC Agg TTA AAC -3′	(21mer)
B:	5′- CCg CTg CAC TgT gAA gCT CT -3′	(20mer)
*(Anti-Sense for G1, G2, G3, G4)		
G5F: (Sense)	5′- AgT gTC ATT TCT TCA AC -3′	(17mer)
G5R: (Anti-Sense)	5′- gTA gTT gTg TCT gCA CAC -3′	(18mer)
G6F: (Sense)	5′- AgA CCA CgT TTC TTg gAg g -3′	(19mer)
G6R: (Anti-Sense)	5′- gTA ggT gTC CAC CgC ggC A -3′	(19mer)

Reagents/Supplies

1. 10x PCR Buffer with 15 mM $MgCl_2$ (PE Applied Biosystems)
   Store long term at -20°C; short term (2 weeks) at 4°C
2. dNTPs, 2 mM total dNTP concentration (0.5 mM each dATP,dTTP,dGTP,dCTP)
   Store long term at -20°C; short term (1 week) at 4°C
3. $MgCl_2$, 25 mM solution
   Store long term at -20°C; short term (2 weeks) at 4°C
4. Dimethyl Sulfoxide (DMSO), store in a light proof bottle, 22°C
5. Primers, stock solutions stored at -20°C;
   25 µM working solutions stored at 4°C
6. Amplitaq DNA Polymerase, 5 U/µl (PE Applied Biosystems)
   Store long term at -20°C; short term (2 weeks) at 4°C
7. Amplitaq Gold DNA Polymerase, 5 U/µl (PE Applied Biosystems)
   Store long term at -20°C; short term (2 weeks) at 4°C
8. Mineral Oil Store at 22°C
9. Water, distilled and deionized
10. 0.2 ml PCR tubes with caps
11. Pipet Tips: 1-20 µl, 10-200 µl, 100-1000 µl for pipetters
12. Combitips: 0.5 ml for repeating pipetter

Instrumentation/Special Equipment

1. Thermal Cycler(s), PE Applied Biosystems Model 9600
2. Thermal Cycler Temperature Verification System, PE Applied Biosystems for GeneAmp PCR Systems: 2400, 9600, 9700
3. Pipetters, 20 µl, 200 µl, 1000µl
4. Repeating Pipetter

Procedure

1. Prepare 25 µg/ml working solutions of each sample to be tested (minimum total volume 150 µl). Thaw reagents. (Note: DNA samples with concentrations of <10 µg/ml or >100 µg/ml may fail to amplify.)
2. Generate an amplification layout of samples to be tested. One or more positive and negative controls should be included for each assay.
   Note: A set of positive controls, which will serve for the amplification and subsequent RFLP step, are selected not only to verify amplification with each specific primer set, but also to ensure that each enzyme used in the RFLP step is making the appropriate cuts.
3. Label 0.2 ml PCR tubes for each group of amplifications with a unique assay number and sample number according to the amplification layout (each thermal cycler run has a specific assay number and each sample within the assay has a unique number).
4. Pipet 20 µl of each DNA sample working solution into its prelabeled tube.
5. Prepare a "master mix" for each set of primers allowing 10% extra for pipetting loss. These master mixes may be prepared as single use aliquots (without the *Taq* polymerase added) and stored at -20°C.

Master Mix:

Reagent	Volume/sample
10x PCR Buffer	10 µl
dNTPs	10 µl
DMSO	5 µl
$MgCl_2$ (25mM)	4-10 µl (see note a below)
Sense primer	1 µl
Anti-sense primer	1 µl
Taq polymerase*	0.6 µl
dH_2O	to 80 µl
	(100 µl total volume)

Notes:

a. $MgCl_2$ (25mM) to add per sample: G1=8 µl, G2=10 µl, G3=6 µl, G4=6 µl, G5=4 µl, G6=6 µl.
b. Primer set G3 should be done in duplicate to generate enough template for the RFLP step.
c. Amplitaq Gold DNA Polymerase is recommended for the G5 primer set.
d. The proportion of master mix reagents and DNA may be decreased depending upon the number of enzyme digests required.

6. Using a repeating pipetter with 0.5 ml combitips, add 80 µl of the appropriate master mix to each 0.2 ml PCR tube.
7. Add one drop of mineral oil to each tube (optional) and cap tubes tightly.
8. Place tubes in the thermal cycler and begin programmed cycling.
   PCR Programs for PE Applied Biosystems Thermal Cycler Model 9600:

Program A (Primer Sets G1, G2, G3, G4, G6)	Program B (Primer Set G5)
a. 94°C, 2 min	a. 95°C, 10 min
b. 10 cycles:	b. 10 cycles:
94°C, 10 sec	94°C, 10 sec
65°C, 1 min	55°C, 1 min
c. 20 cycles	c. 20 cycles:
94°C, 10 sec	94°C, 10 sec
61°C, 50 sec	51°C, 50 sec
72°C, 30 sec	72°C, 30 sec
d. 72°C, 7 min	d. 72°C, 7 min
e. 22°C, hold	e. 22°C, hold

   Note: Thermal cyclers other than the PE Applied Biosystems Thermal Cycler Model 9600 require user validation of the PCR programs listed above.
9. Remove samples from the thermal cycler and store at 4°C or detect amplified DNA using 1.5% agarose gel electrophoresis.

Quality Control

Please refer to the Chapter: "Quality Control and Quality Assurance Monitoring for Molecular Based Methods"

Limitations

Uncharacterized alleles that may have variations or unknown sequences within the primer site may not be amplified.

B. Detection of Amplified DNA Using 1.5% Agarose Gel Electrophoresis

Amplified DNA is visualized by agarose gel electrophoresis and documented with photography.

Reagents/Supplies

1. 20x TBE, (216 g Trizma Base, 110 g Boric acid, 80 ml 0.5M EDTA pH=8, to 1 L).
   Store at 22°C
2. Agarose, any low melting temperature agarose
3. Ethidium bromide (EB, 10 mg/ml)
   Store at 4°C in light-proof bottle
4. Loading buffer, (16 ml 0.04% Bromophenol Blue, 15 ml Glycerol, 4 ml 0.5xTBE, 44 ml dH_2O). Store at 4°C
5. Molecular weight standard (123 base pair ladder).
   Store long term at -20°C; working solution at 4°C
6. dH_2O
7. Pipet tips: 1-20 µl, 10-200 µl
8. Film, Polaroid Black & White Type 667
9. Weigh boats

Instrumentation/Special Equipment

1. 96-well microtiter plates
2. Microwave
3. Thermometer (0-100°C)
4. Magnetic stir bar and stir plate
5. Pan Balance
6. Weigh boats
7. Metal/plastic spatulas
8. Beakers or flasks, (250 ml, 600 ml, 1000 ml)
9. Volumetric flasks (1 L and 2 L)
10. Multichannel pipet (5-50 µl, adjustable)
11. 20 µl pipetter
12. Electrophoresis System.
    Suggested: Owl Scientific Model #A2 or A5
    Gel box dimensions 10 cm H x 28 cm W x 37.5 cm L
    Gel tray 20 cm W x 25 cm L with gasketed end gates
    Combs Model #A2-36C (36 teeth: 3.5 mm wide x 1.0 mm thick)
    Note: if other electrophoresis gel system is used, laboratory must validate the following procedure and parameters with it.
13. Power supply
14. UV transilluminator
15. Polaroid Camera with orange filter.
16. Camera hood(s)

Procedure

1. Prepare 2 liters of 0.5x TBE (50 ml 20x TBE + 1950 ml dH_2O)
2. Place the gel casting tray with the end gates in position on a level surface.
3. Measure 300 ml 0.5x TBE into a beaker or flask (always use a beaker or flask with 1/3 more capacity than the volume being prepared).
4. Add 4.5 g agarose and stir thoroughly.
   Note: Amounts of buffer and agarose may vary with a particular gel tray used.
5. Microwave on high, stirring occasionally, until the mixture comes to a full boil. The solution should be clear and free of any particulates.
6. Cool gel solution to 60°C.
7. Add 3 µl EB and stir well.
8. Pour agarose into the gel tray, insert the combs, and remove and bubbles with a clean pipet tip. Allow 15 minutes for the gel to polymerize.
9. Remove the end gates from the gel tray and place the gel tray in the gel box. Fill the gel box with 0.5x TBE buffer until the buffer is at least 3 mm above the gel.
10. Remove samples from the thermal cycler or refrigerator. Pipet 7 µl from each PCR tube into a corresponding well in a microtiter plate containing 5 µl of loading buffer.
11. Pipet the entire contents (approximately 12 µl) of each microtiter well into a corresponding well in the gel, reserving the first well of each sample set for a molecular weight standard.
12. Add 10 µl of 123 base pair ladder or other standard molecular weight marker into the empty well preceding each set.
13. Close the gel box, plug the electrodes into the power supply, and run the gel for 20 minutes (negative to positive electrode) at 300 volts or until the loading buffer migrates 2-3 cm (time and voltage varies with each gel apparatus used).
14. Turn off the power supply, transfer the gel to a UV transilluminator, and take a photograph.
15. Document the positive amplifications by highlighting the corresponding amplication number on the amplification layout.
16. Store the samples at 4°C or 22°C until the appropriate enzyme digestions can be performed.
    Note: The amplified DNA will start to degrade after prolonged storage (>2 months). Interpretation of RFLP patterns will be compromised by degraded DNA.

Quality Control

1. The positive control lane must contain a band of predicted size and must not be significantly weaker than any other amplified sample of that primer mix. If these criteria are not met, the amplification should be repeated.
2. The negative control lane must not reveal any amplified product.
3. Only one band of the correct size must be present. If more than one amplified product is detected, the restriction enzymes used in the RFLP assay may produce fragments that interfere with the interpretation of alleles present.

Limitations

Cloudy patch formation in the gel due to impurities or inadequate heating time, or bubbles in the gel, will cause abnormal migration of samples and inaccurate banding patterns.

C. DRB1 Restriction Enzyme Digestions

Reagents/Supplies

1. Group specific amplified DNA samples
2. Restriction enzymes, 10x reaction buffers, 100x BSA (New England Biolabs)
   Enzymes are kept at -20°C, buffers and BSA at -20°C (long term); 4°C (short term).
3. dH_2O
4. 96-well microtiter plates and plate sealers
5. Pipet tips: 1-20 µl, 10-200 µl, 100-1000 µl
6. 0.05 ml combitips

Instrumentation/Special Equipment

1. Incubators (37°C & 60°C)
2. Multichannel pipet (5-50 µl, adjustable)
3. Pipetters (20 µl, 200 µl, 1000 µl)

Procedure

1. Determine the groups requiring enzyme digestions:

ENZYME	G1	G2	G3	G4	G5	G6(none)
*Ava*II		x		x		
*Bbv*I			x			
*Bsr*BI			x			
*Bst*NI*			x			
*Bst*UI*	x	x	x	x		
*Cfo*I		x	x	x		
*Fok*I	x	x	x			
*Fnu*4HI		x				
*Hae*III	x					
*Hin*fI		x	x	x	x	
*Hph*I	x	x	x	x		
*Mn*lI			x	x		
*Msp*AI				x		
*Nci*I			x			
*Pst*I	x		x			
*Rsa*I			x			
*Sfa*NI		x				

   *Incubation temperature = 60°C instead of 37°C

   Note: The number of enzymes may vary with the level of resolution required.
2. Place positive amplified samples by group (DRB1-G1, G2, etc) into a rack and assign a well number for each sample (each well number corresponds to one amplified DNA sample per group).
3. Label microtiter plate with unique digestion number, group number, enzymes (y-axis), and well number (x-axis). Enzyme digestions requiring the same temperature may be loaded onto the same plate.
4. Determine the number of amplified DNA samples (including control) to be digested with each enzyme. Prepare a master mix for each enzyme (allow 10% extra for pipetting loss) and pipet 5 µl of each mix into the appropriate wells of the labeled microtiter plate(s):

   Master Mix:

Reagent	Volume/sample
Enzyme	3-5 units
10x buffer	2 µl
100x BSA (if necessary)	0.1 µl
dH_20	to 5 µl

5. Pipet 11-12 µl of amplified DNA into appropriate well(s) and mix well.
6. Cover microtiter plate with a plate sealer and incubate for 1 hour at the optimal temperature listed for each enzyme by the manufacturer.
7. Remove tray(s) from the incubator(s) after incubation and store at 4°C or detect PCR-RFLP patterns using 4% agarose gel electrophoresis.

D. Detection of PCR-RFLP Patterns Using 4% Agarose Gel Electrophoresis

PCR-RFLP patterns are determined by 4% agarose gel electrophoresis and documented with photography.

Reagents/Supplies

1. 96-well microtiter plates containing group specific amplified DNA samples digested with appropriate restriction enzymes
2. 20x TBE, (216 g Trizma Base, 110 g Boric acid, 80 ml 0.5M EDTA pH=8, to 1 L).
   Store at 22°C
3. Metaphor Agarose, FMC Bioproducts
4. Molecular weight standard ØX174RFII DNA *Taq*αI digest (New England Biolabs)
5. Ethidium bromide (EB, 10mg/ml)
   Store at 4°C in light-proof bottle
6. Loading buffer, (16 ml 0.04% Bromophenol Blue, 15 ml Glycerol, 4 ml 0.5x TBE, 44 ml dH_2O). Store at 4°C
7. Distilled water
8. Pipet tips: 1-20 µl, 10-200 µl
9. Film, Polaroid Black & White Type 667
10. Weigh boats

Instrumentation/Special Equipment

1. Microwave
2. Thermometer (0-100°C)
3. Magnetic stir bar and stir plate
4. Pan Balance
5. Weigh boats
6. Metal/plastic spatulas
7. Beakers (250 ml, 600 ml, 1000 ml)
8. Volumetric flasks (1L and 2L)
9. Multichannel pipet (5-50 µl, adjustable)
10. 20 µl pipetter
11. Electrophoresis System.
    Suggested: Owl Scientific Model #A2 or A5
    Gel box dimensions 10 cm H x 28 cm W x 37.5 cm L
    Gel tray 20 cm W x 25 cm L with gasketed end gates
    Combs Model #A2-36C (36 teeth: 3.5 mm wide x 1.0 mm thick)
12. Power supply
13. UV transilluminator
14. Polaroid Camera with orange filter
15. Camera hood(s)

Procedure

1. Prepare 2 liters of 0.5x TBE (50 ml 20x TBE + 1950 ml dH_2O)
2. Place the gel casting tray with the end gates in position on a level surface.
3. Measure 400 ml 0.5x TBE into a 600 ml beaker (for each 4% gel)
4. Slowly stir in 16 g Metaphor agarose
   Note: Although the amounts of buffer and agarose may vary with a particular gel tray used, the well capacity for this 4% gel should be no less than 20 µl.
5. Microwave on high for 2-3 minutes. Stir and continue microwaving until the solution comes to a full boil and the agarose completely dissolves. The solution should be clear and free of any particulates.
6. Cool gel solution to 60°C.
7. Add 4 µl EB and stir well.
8. Pour agarose into the gel tray, insert the combs, and remove bubbles with a clean pipet tip. Allow 15 minutes for the gel to polymerize.
9. Remove the end gates from the gel tray and place the gel tray in the gel box. Fill the gel box with 0.5x TBE buffer until the buffer is at least 3 mm above the gel.
10. Add 5 µl chilled loading buffer to the microtiter plate wells containing the enzyme digestions.
11. Pipet the entire contents of each microtiter well (approximately 20 µl) into a corresponding well in the gel, reserving the first well of each set for the ØX174 *Taq*αI marker.
    Note: Loading samples digested with the same enzyme into adjacent lanes simplifies the interpretation of banding patterns and makes it easier to detect minute variances in fragment sizes
12. After all the enzyme digestions have been loaded onto the gel, pipet 18 µl of the ØX174RFII DNA *Taq*αI digest size marker into the well immediately preceding each set of enzyme digestions.
13. Close the gel box, plug the electrodes into the power supply, and run the gel (negative to positive electrode) for 1-1.5 hr at 200 volts or until the loading buffer migrates 4 cm (time and voltage varies with each gel apparatus used).

Note: Since Metaphor is an intermediate melting temperature agarose, these 4% gels may melt at higher voltages

14. Turn off the power supply, transfer the gel to a UV transilluminator, and take a photograph.

Results

The predicted restriction fragment sizes for each amplicon and enzyme combination are determined by computer analysis.[11] HLA allele sequences are obtained via internet at www.ebi.ac.uk/home.html and by WHO Nomenclature Committee publications[12,13] (visit the ASHI website for links to these and other molecular data of interest). If necessary, the restriction fragment sizes are manually confirmed and adjusted, based on the published off-set of the recognition sites and cut sites (New England Biolabs 1999-2000 Catalog, New England Biolabs, Inc., Beverly, MA).

Tables 2a-2f illustrate the size of electrophoretically detected fragments found when the indicated alleles are present and amplicons are digested with the indicated enzymes. Fragments which are <20 base pairs have been omitted from the worksheets because they are not reliably visible. The net pattern of detected bands in heterozygotes is a combinatorial (logical "or" function) of the patterns for the two respective alleles found in a given individual. An enzyme analysis worksheet can be generated so that technologists can simply identify a band pattern in the gel, look that pattern up on the worksheet, and determine which genotype(s) is consistent with that pattern. The interpretation of each sample is facilitated by the use of these enzyme analysis sheets (Tables 2a-2f) by highlighting which banding pattern is present for each enzyme.

Limitations

1. Alleles with sequence variations that are not within a restriction enzyme's recognition site will not be detected.
2. Computer modeling of expected RFLPs requires complete sequence definition of each amplicon in order to identify restriction sites and predict fragment sizes.
3. Certain DRB1 alleles give rise to identical RFLP patterns. PCR-RFLP used in combination with PCR-SSP and PCR-SSOP has proven to be an alternative for solving these problems. Please refer to the appropriate chapters for more information.
4. In some unique cases all of the predicted fragments may not be present because a cut in one location may inhibit a neighboring cut site. An example of this would be the MnI pattern for the DRB1*0404 amplicon.
5. Many of the heterozygous ambiguities within an amplification group may be resolved by separating the 2 alleles with additional primer sets that specifically amplify alleles for the glycine/valine polymorphism at codon 86.[4] The enzyme HphI detects this polymorphism.
6. As with all techniques, there are some indistinguishable heterozygote combination patterns. The cis/trans orientation of detected motifs cannot be unequivocally assigned without family segregation studies or cloning of the alleles.

References

1. Wake C, Long E, Mach B, Allelic polymorphism and complexity of the genes for HLA-DR β-chains: Direct analysis by DNA-DNA hybridization. *Nature* 300: 372-374, 1982.
2. Mullis K, Faloona F, Scharf S, Saiki R, Horn G, Erlich H, Specific enzymatic amplification of DNA in vitro: the polymerase chain reaction. Cold Spring Harb Symp Quant *Biol* 51:263-273, 1986.
3. Cereb N, Maye P, Lee S, Kong Y, Yang SY, Locus-specific amplification of HLA class I genes from genomic DNA: locus-specific sequences in the first and third introns of HLA-A, -B, and -C alleles. *Tissue Antigens* 45:1-11, 1995.
4. Olerup O, Zetterquist H, HLA-DR typing by PCR amplification with sequence-specific primers (PCR-SSP) in 2 hours: analternative to serological DR typing in clinical practice including donor-recipient matching in cadaveric transplantation. *Tissue Antigens* 39:225-235, 1992.
5. Santamaria P, Boyce-Jacino MT, Lindstrom AL, Barbosa JJ, Faras AJ, Rich SS, HLA class II "typing": direct sequencing of DRB, DQB, and DQA genes. *Hum Immunol* 33:69-81, 1992.
6. Yunis I, Salazar M, Yunis EJ, HLA-DR Generic Typing by AFLP. *Tissue Antigens* 38:78-88, 1991.
7. Salazar M, Yunis I, Alosco SM, Chopek M, Yunis EJ, HLA-DPB1 allele mismatches between unrelated HLA-A, B, C, DR (generic) DQA1-identical unrelated individuals with unreactive MLC. *Tissue Antigens* 39:203-208, 1992.
8. Salazar M, Yunis JJ, Delgado MB, Bing D, Yunis EJ, HLA-DQB1 allele typing by a new PCR-RFLP method: Correlation with a PCR-SSO method. *Tissue Antigens* 40:116-123, 1992.
9. Granja CB, Salazar M, Bozon V, Ohashi MK, Yunis EJ, Complete allele typing of DR2-DRB1 by a combination of PCR-RFLP and PCR-SSP. *Tissue Antigens* 47:80-84, 1996.
10. Bodmer JG, Marsh SGE, Albert ED, Bodmer WF, Bontrop RE, Charron D, Dupont B, Erlich HA, Fauchet R, Mach B, Mayr WR, Parham P, Sasazuki T, Schreuder GM, Strominger JL, Svejgaard A, Terasaki PI, Nomenclature factor of the HLA system, 1996. *Tissue Antigens* 49:297-321, 1997.
11. DNA Inspector IIe, Manual and Tutorials, Textco, Inc., West Lebanon, NH.
12. Mason PM, Parham PM, HLA class I sequence, 1998. *Tissue Antigens* 51:417-466, 1998
13. Marsh SGE, HLA class II sequence, 1998. *Tissue Antigens* 51:477-507, 1998

Table 1. List of DRB1 Alleles Recognized by the PCR-RFLP Method Proposed

DRB1*0101
DRB1*01021
DRB1*01022
DRB1*0103
DRB1*0104

DRB1*1501=*15031
DRB1*15021
DRB1*15022
DRB1*1504
DRB1*1505
DRB1*1506
DRB1*16011
DRB1*16012
DRB1*16012=*16031
DRB1*16021
DRB1*16022
DRB1*1604
DRB1*1605
DRB1*1607
DRB1*1608

DRB1*0301
DRB1*0302=*0311
DRB1*0303
DRB1*0304
DRB1*0305
DRB1*0306
DRB1*0307
DRB1*0308
DRB1*0309
DRB1*0310

DRB1*04011=*0416
DRB1*04012
DRB1*0402
DRB1*0403
DRB1*0404
DRB1*04051=*0424
DRB1*04052
DRB1*0406
DRB1*0407
DRB1*0408
DRB1*0409
DRB1*0410
DRB1*0411
DRB1*0412
DRB1*0413
DRB1*0414
DRB1*0415
DRB1*0417
DRB1*0418
DRB1*0419

DRB1*0420
DRB1*0421
DRB1*0422
DRB1*0423
DRB1*0425

DRB1*11011=*11012=*1127
DRB1*11013
DRB1*1102=*1121
DRB1*1103
DRB1*1104=*1106
DRB1*1105
DRB1*1107
DRB1*1108
DRB1*1109=*1110
DRB1*1111
DRB1*1112=*1124=*1128
DRB1*1113
DRB1*1114
DRB1*1115
DRB1*1116
DRB1*1117
DRB1*1118
DRB1*1119
DRB1*1120
DRB1*1122
DRB1*1123
DRB1*1125
DRB1*1126
DRB1*1129
DRB1*1130
DRB1*1131

DRB1*1201=*1205
DRB1*12021
DRB1*12022
DRB1*12032
DRB1*1204

DRB1*1301=*1315=*1316
DRB1*1302=*1331
DRB1*1303=*1312
DRB1*1304
DRB1*1305
DRB1*1306=*1310
DRB1*1307
DRB1*1308=*1319
DRB1*1309
DRB1*1311
DRB1*1313
DRB1*1314
DRB1*1317
DRB1*1318

DRB1*1320
DRB1*1321
DRB1*1322
DRB1*1323
DRB1*1324
DRB1*1325
DRB1*1326
DRB1*1327
DRB1*1328
DRB1*1329
DRB1*1330
DRB1*1332

DRB1*1401=*1426
DRB1*1402=*1409
DRB1*1403
DRB1*1404=*1423=*1428
DRB1*1405
DRB1*1406=*1420=*1429
DRB1*1407
DRB1*1408
DRB1*1410
DRB1*1411
DRB1*1412
DRB1*1413
DRB1*1414
DRB1*1415
DRB1*1416
DRB1*1417
DRB1*1418
DRB1*1419
DRB1*1421
DRB1*1422
DRB1*1424
DRB1*1425
DRB1*1427
DRB1*1430

DRB1*0701

DRB1*0801
DRB1*0802=*0807
DRB1*08031
DRB1*08032
DRB1*0804
DRB1*0805
DRB1*0806
DRB1*0808
DRB1*0809
DRB1*0810
DRB1*0811
DRB1*0812
DRB1*0813

DRB1*0814
DRB1*0815
DRB1*0816
DRB1*0817

DRB1*09012

DRB1*1001

Table 2a: DRB1-G1 Enzyme Analysis (alleles in numeric order)

Allele	Fok I			Hph I			Pst I			BstUI		
	2	1		1	1	1	2	2		2	2	
	6	7	9	4	1	0	6	1	4	6	0	6
	1	1	0	1	9	9	1	5	6	1	0	1
*0101	|			|		|		|	|		|	|
*01021	|			|	|		|				|	|
*01022	|			|	|		|			|		
*0103		|	|	|		|		|	|		|	|
*0104	|			|	|			|	|		|	|

Table 2b: DRB1-G2 Enzyme Analysis (alleles sorted by CfoI patterns)

Allele	Cfo I						Hph I				FokI			Hae III				AvaII			HinfI			BstUI			SfaNI		
	1						2	1	1	1	2	1		1	1			2	1		2	1		2	2		2	1	1
	6	9	9	9	6	6	6	4	1	0	6	7	8	4	1	6	5	6	7	8	6	7	8	6	0	6	6	5	1
	6	9	7	5	9	5	1	1	9	9	1	9	2	5	6	4	2	1	6	5	1	4	7	1	0	1	1	1	1
*1501=1503			|	|	|			|	|			|	|		|	|	|				|	|	|	|		|		
*1504			|	|	|			|	|		|			|		|	|				|	|	|	|		|		
*1505			|	|	|			|	|		|			|		|	|				|	|	|	|		|		
*15021			|	|	|			|		|		|	|		|		|	|				|	|	|	|		|		
*15022			|	|	|			|		|		|	|		|		|	|				|	|	|			|	|	
*1506			|	|	|		|				|	|		|	|	|				|	|		|	|	|		|		
*16011		|		|		|		|		|			|	|			|				|	|		|	|	|		|		
*1608		|		|		|		|		|			|	|			|			|				|	|	|		|		
*16021		|		|		|		|		|			|	|				|	|				|	|	|		|		
*1605		|		|		|		|			|	|	|	|			|				|	|	|		|		
*1607		|		|		|		|			|	|	|	|			|		|				|	|	|		|		
*16022	|			|			|		|	|			|		|	|			|	|			|	|	|		|		
*16012=1603	|			|			|		|	|			|		|	|			|	|			|	|	|		|		
*1604	|			|			|		|	|			|		|	|			|	|		|			|				|		

Table 2c: DRB1-G3 Enzyme Analysis (alleles sorted by RSA I patterns)

| Allele | Rsa I | | | | | | | | | | | | | | | | | BstN I | | | | | | | | | | | | | | Cfo I | | | | | | | | | | | | | Fok I | | | Nci I | | | | | | | | | | | Hph I | | | | | BstUI | | | | | | | | | | |
|---|---|---

Table 2d: DRB1-G3 Enzyme Analysis Continued (alleles in numeric order)

Allele	Mnl I														Fnu4HI								Bbv I				Hinf I					BsrB I						Pst I		
	1	1	1	1	1										1	1	1	1					2	2	1	1	2	1	1	1		2	1	1				2	2	
	8	8	1	0	0	8	8	7	7	6	6	4	3		4	3	1	0	6	5	5	4	6	4	5	0	6	7	6	0	9	1	4	4	7	6	5	6	2	4
	3	0	4	9	7	5	2	6	4	9	6	0	8		8	5	1	4	3	6	5	8	6	6	5	4	6	4	1	5	2	6	7	1	5	9	0	6	0	6
*0301	|			|			|		|					|		|																				|	|
*0302	|			|			|		|					|		|																				|	|
*0303	|			|			|		|					|		|																				|	|
*0304	|			|			|		|					|		|										|			|		|					|	|
*0305	|			|			|		|					|		|									|					|					|	|
*0306	|			|			|		|					|		|									|					|					|	|
*0307	|			|			|		|					|		|									|					|					|	|
*0308							|		|	|		|		|		|									|					|					|	|
*0309	|			|			|		|					|		|									|					|					|	|
*0310	|			|			|		|					|	|							|	|	|					|					|	|
*0311	|			|			|		|					|		|									|					|					|	|
*1101,27			|				|			|					|				|	|					|					|					|	|
*11013			|				|			|					|				|	|					|					|					|	|
*1102,21			|				|			|					|				|	|							|			|	|				|	|
*1103			|				|			|					|				|	|					|					|	|				|	|
*1104,6			|				|			|					|				|	|					|					|					|	|
*1105			|				|			|					|				|	|					|					|					|	|
*1107							|		|	|	|			|					|					|					|					|	|
*1108							|		|	|	|							|	|					|					|					|	|
*1109,10			|				|			|					|				|	|					|					|					|	|
*1111			|				|			|					|				|	|							|			|	|				|	|
*1112,24,28			|				|			|					|				|	|					|					|					|	|
*1113							|	|	|	|	|	|		|					|	|							|	|				|		
*1114			|				|			|					|				|	|						|			|	|				|	|
*1115			|				|			|	|			|				|	|					|					|					|	|
*1116			|				|			|					|				|	|						|			|	|				|	|
*1117							|			|	|	|	|	|		|					|	|						|	|				|		
*1118			|				|			|					|				|	|					|					|					|	|
*1119			|				|			|					|				|	|					|					|					|	|
*1120			|				|			|					|				|	|						|			|	|				|	|
*1123			|				|			|					|	|				|	|					|					|					|	|
*1125			|				|			|					|	|				|	|					|					|					|	|
*1126							|	|	|	|	|	|		|					|	|					|					|					|	|
*1129			|				|			|					|					|	|						|			|					|					|	|
*1130			|				|			|						|					|					|					|					|					|	|
*1131			|				|			|					|					|	|					|					|					|					|	|
*1201,5	|						|								|					|	|							|	|					|					|	|		
*12021	|						|								|					|	|							|	|					|					|	|		
*12022	|						|								|					|	|							|	|					|					|			|	|
*12032	|						|								|					|	|							|	|					|					|			|	|
*1204			|				|			|					|					|	|					|					|					|	|		
*1301,15,16	|						|								|					|	|							|	|					|					|	|		
*1302,31	|						|								|					|	|							|	|					|					|	|		
*1303,12	|						|								|					|	|							|						|					|	|		
*1304	|						|								|					|	|							|	|					|					|	|		
*1305	|						|								|					|	|							|						|					|	|		
*1306,10	|						|								|					|	|							|						|					|	|		
*1307	|						|								|					|	|							|						|					|	|		
*1308,19	|						|								|					|	|							|	|					|					|	|		
*1309	|						|								|					|	|							|						|					|	|		
*1311	|						|								|					|	|							|						|					|	|		
*1313	|						|								|	|				|								|						|					|	|		
*1314	|						|								|					|	|							|						|					|	|		

Table 2d: continued

Allele	MnlI														Fnu4HI								BbvI				HinfI					BsrBI						PstI		
	1	1	1	1	1										1	1	1	1					2	2	1	1	2	1	1	1		2	1	1				2	2	
	8	8	1	0	0	8	8	7	7	6	6	4	3		4	3	1	0	6	5	5	4	6	4	5	0	6	7	6	0	9	1	4	4	7	6	5	6	2	4
	3	0	4	9	7	5	2	6	4	9	6	0	8		8	5	1	4	3	6	5	8	6	6	5	4	6	4	1	5	2	6	7	1	5	9	0	6	0	6
*1317	|						|							|				|	|		|				|					|		|	|		|	|
*1318	|						|							|		|					|				|				|				|		|	|
*1320				|			|		|					|				|	|		|				|					|		|	|		|	|
*1321	|						|							|				|	|		|				|				|				|		|	|
*1322	|						|							|				|	|		|				|					|		|	|		|	|
*1323	|						|							|				|	|		|				|					|		|	|		|	|
*1324	|						|							|				|	|		|				|					|		|	|		|	|
*1325				|			|		|					|				|	|		|				|				|				|		|	|
*1326	|						|							|				|	|		|				|				|				|		|	|
*1327	|						|							|				|	|		|				|					|		|	|		|	|
*1328	|						|							|				|	|		|				|					|		|	|		|	|
*1329				|			|		|					|				|	|		|				|					|		|	|		|	|
*1330	|						|							|			|	|		|				|					|				|		|	|
*1332	|						|							|				|	|		|				|					|		|	|		|	|
*1401,26				|	|		|			|				|		|						|	|		|				|	|			|		
*1402,9				|	|		|	|	|					|				|	|		|		|		|				|		|	|
*1403				|			|		|					|	|					|				|					|				|		|	|
*1404,23,28				|	|		|			|				|		|						|	|		|				|	|			|		
*1405				|	|		|			|				|	|					|				|					|	|			|		
*1406,20,29				|	|		|	|	|					|				|	|		|				|				|				|	|
*1407				|	|		|			|				|		|						|	|		|				|	|			|		
*1408				|	|		|		|					|	|					|				|					|	|			|		
*1411								|		|	|	|	|		|					|				|					|	|			|		
*1412				|			|	|						|	|					|				|					|				|		|	|
*1413				|	|		|	|	|					|				|	|		|		|					|				|		|	|
*1414				|	|		|			|				|	|					|				|						|	|			|		
*1415	|						|							|	|					|				|					|				|		|	|
*1416	|						|							|				|		|		|	|		|					|		|		|	|
*1417				|	|		|	|	|					|				|	|		|		|					|				|		|	|
*1418				|	|		|			|				|	|					|				|						|	|			|		
*1419				|			|		|					|				|	|		|				|					|				|		|	|
*1421				|			|		|					|				|	|		|				|					|				|		|	|
*1422	|						|							|				|		|		|	|		|					|				|		|	|
*1424	|						|							|				|	|		|				|					|				|		|	|
*1425	|						|							|				|		|		|	|		|					|				|		|	|
*1427	|						|							|	|					|				|					|				|		|	|
*1430				|	|		|	|	|					|				|	|		|		|					|				|		|	|
*0801	|						|							|	|					|				|					|				|		|	|
*0802,7	|						|							|	|					|				|					|				|		|	|
*08032	|						|							|	|					|				|					|				|		|	|
*08041,2,3	|						|							|	|					|				|					|				|		|	|
*0805	|						|							|				|	|		|				|					|				|		|	|
*0806	|						|							|	|					|				|					|				|		|	|
*0808	|						|							|		|					|		|	|		|					|				|		|	|
*0809	|						|							|	|					|				|					|				|		|	|
*0810,12	|						|							|	|					|				|					|				|		|	|
*0811	|						|							|		|					|		|	|		|					|				|		|	|
*0813				|			|		|					|	|					|				|					|				|		|	|
*0814	|			|											|	|					|				|					|				|		|	|
*0815	|			|											|	|					|				|					|				|		|	|
*0816	|	|				|	|							|	|					|				|					|				|		|	|
*0817	|						|							|	|					|				|					|				|		|	|

Table 2e: DRB1-G4 Enzyme Analysis (alleles sorted by MnlI patterns)

Allele	MnlI										HphI						MspA1I			CfoI					HinfI				AvaII			BstUI		
	1	1	1	1	1						1	1	1				2	2		1	1	1			2	2	1		2	1		2	2	
	8	8	1	0	0	7	7	7	6	6	4	1	0	7	7	5	5	0	5	6	5	1	9	5	6	4	7	8	6	7	8	6	0	6
	8	3	4	9	7	9	6	4	9	6	2	9	9	9	2	1	7	1	6	6	3	0	7	6	3	6	4	9	3	8	5	3	2	1
***04011,16**				|		|		|				|		|			|	|			|						|	|			|	|
***0409**				|		|		|				|		|			|		|	|	|		|					|	|			|	|
***0413**				|		|		|			|			|			|	|			|		|		|					|	|			|	|
***0421**				|		|		|				|		|			|	|			|				|	|					|	|			|	|
***0422**				|		|		|			|			|		|					|				|	|					|	|			|	|
***04012**	|							|				|		|				|	|			|				|					|	|			|	|
***0402**		|				|					|			|				|	|			|				|					|				|	|
***0412**		|				|					|			|		|				|	|	|		|				|				|						
***0414**		|				|						|		|				|	|			|				|					|	|			|	|
***0418**		|				|					|			|		|				|			|				|					|				|	|
***0425**		|				|					|		|	|		|				|			|				|					|						
***0403**				|	|	|				|			|			|				|			|				|					|	|					
***0406**				|	|	|				|			|			|				|			|						|	|	|			|	|
***0407**				|	|	|				|		|		|		|				|			|				|					|	|			|	|
***0411**				|	|	|				|		|			|				|	|	|		|				|					|	|			|	|
***0417**				|	|	|				|			|			|				|	|	|		|				|					|	|			|	|
***0420**				|	|	|				|			|			|				|			|						|	|	|			|	|
****1410***				|	|	|				|	|					|				|			|				|					|	|			|	|
***0404**				|	|	|	|	|			|			|			|	|			|				|					|	|			|	|
***04051,24**				|	|	|	|	|				|		|			|	|				|	|			|				|				|	|
***04052**				|	|	|	|	|				|		|			|	|				|				|				|				|	|
***0408**				|	|	|	|	|				|		|			|	|				|				|				|				|	|
***0410**				|	|	|	|	|			|			|			|	|				|	|			|				|				|	|
***0419**				|	|	|	|	|				|		|			|	|				|				|				|				|	|
***0423**				|	|	|	|	|			|			|			|	|				|	|			|				|				|	|
***0415**			|			|			|			|			|			|	|				|				|				|				|	|
****1122***			|			|			|		|		|				|	|				|				|				|				|				|	|

Table 2f: DRB1-G5 Enzyme analysis

Allele	HinfI			
	1			
	3	9	7	4
	5	0	4	5
*0701		|	|	|
*09012	|		|	

Enzyme – Linked DNA Oligotyping Performed In Microtiter Plates (ELDOT)

Aloke Mohimen and Marcelo Fernández-Viña

Purpose

Correct typing of HLA antigens is an important prerequisite for diverse fields of study, including antigen presentation, donor-recipient selection in clinical transplantation, population origins and migration, genetic susceptibility to diseases, paternity testing and forensic investigation. Presently, one of the methods of choice for HLA typing involves DNA amplification by Polymerase Chain Reaction (PCR) combined with hybridization of sequence specific oligonucleotide probes which identify all alleles, including micro polymorphic differences.

Here we describe a simplified genotyping procedure to type HLA-A and HLA-DR alleles. In contrast to classic hybridization in membranes, this procedure is based on the detection of the presence of a gene sequence through DNA-probe hybridization by an Enzyme Linked Immunosorbent assay performed in wells of microtiter plate. Briefly the method consists of immobilization of biotin labeled PCR amplified DNA to avidin coated wells of a 96-well microtiter plate. The unlabelled sense strand is removed during a brief 0.5N Sodium Hydroxide wash. Subsequent hybridization to a horseradish peroxidase labeled oligonucleotide probe, and spectrophotometric detection of a soluble color using a peroxidase substrate. Using this procedure, high resolution DNA typing can be performed on single or multiple samples with a rapid turn- around time, making it feasible for clinical purposes and/or automation.

Specimens

This procedure is based upon the use of genomic DNA templates, which can be prepared using a variety of methods, some of which are described in the chapter on DNA Isolation Methods in this Manual (chapter V.A.1). Please note that many factors can have an effect on the amplification of templates by PCR isolated from blood, including age, storage condition and the anti coagulant used for collecting blood.

Unacceptable specimens are usually those that yield low amounts of amplified products or total result in failure of amplification (again, see chapter V.A.1).

Protocol

The ELDOT protocol is divided into 5 components, which are listed below.

A. PCR amplification of HLA-A and HLA-DRB
B. Procedure for amplification confirmation
C. Coating of microtiter plates
D. Conjugation of oligonucleotide probes to horseradish peroxidase
E. Procedure for ELDOT assay and data interpretation

A. PCR Amplification of HLA-A and HLA-DRB

Purpose

This procedure involves the in vitro amplification of HLA-A and HLA-DRB region using specific oligonucleotide primers. The primers bind to opposite ends of the gene and Taq polymerase directs synthesis of a new strand using dNTP as building blocks. There are three major steps: 1) Denaturation- making the DNA single stranded (high temperature), 2) Annealing- binding of primers (lower temperature) and 3) Extension- strand synthesis (intermediate temperature). These steps are repeated for thirty cycles to provide an exponential yield.

Instrumentation

1. Thermal cycler
2. Micro pipettes and tips.

Reagents and Supplies

1. 10X PCR Buffer (100mM Tris-HCl, pH 8.3, 500 mM KCl.)
2. 25 mM $MgCl_2$
3. dNTP stock (10mM each of dATP,dCTP,dGTP,dTTP, pH 7.0)
4. Primers-each diluted to 20 µM:

HLA –A Primer

1) 5A.2: 5′ – CC CAG ACG CCG AGG ATG GCC G-3′
2) 3A.2biotin: 5′-Biotin-GCA GGG CGG AAC CTC AGA GTC ACT CTC T- 3′

HLA-DRB Primer

1) DRB 5′.2: 5′- CGT GTC CCC ACA GCA CGT T-3′
2) DRB 3′.2 biotin: 5′- Biotin- CCG CTG CAC TGT GAA GCT CT-3′

5. Dimethyl Sulfoxide (DMSO)
6. Taq Polymerase-5 Units/µl
7. Water: sterile distilled and deionized.
8. PCR tubes with caps: Tubes used for thermal cycling should achieve good contact with thermalcycler wells and have superior heat exchange.

Procedure

a. Preparation of Master Mixture for Amplification

1. Master mixture for 96 amplification of HLA-A.
   Transfer the reagents listed below to a 15 ml tube
   1000 µl of 10X PCR Buffer
   250 µl of DMSO
   200 µl of dNTP
   600 µl of $MgCl_2$
   100 µl of 5A.2 Primer
   100 µl of 3A.2 biotin Primer
   50 µl of Taq Polymerase
   Sterile distilled water to make a final volume of 10 ml.
2. Master mixture for 96 amplification of HLA-DRB
   Transfer the reagents listed below to a 15-ml tube
   1000 µl of 10X PCR Buffer
   600 µl of $MgCl_2$
   200 µl of dNTP
   100 µl of DRB 5′.2 Primer
   100 µl of DRB 3′.2 biotin Primer
   50 µl of Taq Polymerase
   Sterile distilled water to make a final volume of 10 ml.

   Note: Prepare the mixture on ice. Vortex the whole mixture before use in the next step.

b. Amplification Reaction Set up

1. Dispense 90 µl of the master mixture into each PCR tube.
2. Add 10 µl of template DNA to each tube.
3. Cap each tube after adding DNA.

c. PCR Amplification

Use the following Program for HLA-A:

Step		Temperature	Time
Initial Heating		96°C	2 min.
30 cycles of:	Denaturation	94°C	22 Sec
	Annealing	65°C	50 Sec
	Extension	72°C	30 Sec
Final extension		72°C	10 min

Use the following Program for HLA-DRB:

Step		Temperature	Time
Initial Heating		96°C	1 min
29 cycles of	Denaturation	95°C	1min
	Annealing	56°C	1min
	Extension	72°C	1 min
Final extension	72°C	10 min	

Store all samples at 4°C after completion of PCR reaction.

B. Procedure for Confirmation of Amplification

Purpose

The purpose of this procedure is to quantitate the amplicon DNA to determine if the PCR amplification was successful. It can be done either by electrophoresis of amplified DNA or by a fluorescent assay using PicoGreen. This procedure is a rapid but very sensitive method. A small aliquot of each amplification product will be mixed with the PicoGreen dye. After a short incubation, sample fluorescence will be measured using a fluorometer, indicating the quantity of double stranded DNA (dsDNA) present. For the spectrophotometric determination of dsDNA, refer to chapter V.A.1.

Instrumentation and Supplies

1. Fluoroscan II or other flurometer capable of reading micro titer plates
2. Microfluor plates
3. PicoGreen dye (Molecular Probes #P7581)
4. TE buffer
5. Pipettors
6. Pipette tips
7. 50 ml plastic tubes
8. Pipettes
9. Aluminum foil

Procedure

a. Fluoroscan II Operation

1. Turn the Fluorscan II flurometer at least 1 hr. before starting the procedure.
2. Set both the excitation filter and the emission filter to setting 2. The excitation filter should be 485nm and the emission filter should be 538nm.

b. PicoGreen Preparation

1. Bring the PicoGreen reagent to room temperature (approximately 1 hour).
2. Prepare a working solution of the PicoGreen dye by diluting it 1:20 in TE buffer. PicoGreen dye is sold as a solution in DMSO. In order to prepare enough reagent for two 96 well plates of PCR amplification, add 100 µl PicoGreen reagent to 19.9 ml of TE buffer in a plastic 50 ml tube.
3. Wrap the tube containing the working solution of PicoGreen dye in aluminum foil to protect from light.

c. DNA Detection

1. Add 90 µl of TE buffer to each well of a microfluor plate.
2. Add 10 µl of PCR amplified product to the corresponding wells of the microfluor plate and mix.
3. Add 100 µl of working solution of the PicoGreen reagent to each well and mix by tapping gently.
4. Cover the plate with foil and incubate for 5 minutes at room temperature.
5. After incubation, measure the sample fluorescence using the Fluoroscan II
6. The fluorescence reading of each amplicon must be at least three times that of the PCR negative control reading. Higher control values suggest the presence of primer- dimer contamination in the amplicon.
   Note: PicoGreen should be stored frozen at -20°C. It must be brought to room temperature before use and it must be protected from light. Currently there are no data on the toxicity or mutagenicity of the PicoGreen reagent. However, since it binds to double stranded DNA, it should be treated as a possible mutagen. In addition, the reagent contains DMSO, which is known to facilitate the uptake of organic molecules into tissue. Therefore caution should be exercised when using this reagent. Personal protective equipment is recommended in the handling of both reagents.

C. Coating of Microtiter Plates

Purpose

The preparation of microtiter plates to make them suitable for immobilization of amplicons on a solid support (the microtiter plates) and to provide a convenient format for hybridization with a panel of oligonucleotide probes.

Instrumentation and Supplies

1. Micro titer plates- Enhanced protein binding properties (Pierce #15040)
2. Multichannel pipettes
3. Pipette tips
4. Microplate auto washer
5. Enhanced protein binding 96 well micro titer plates.
6. Avidin from egg white (chromatographically purified)
7. Carbonate-Bicarbonate buffer, (100mM Sodium Carbonate-Bicarbonate, pH 9.5)
8. Tris-Saline Buffer (100mM Tris HCl, pH 7.4; 150mM NaCl)
9. Gelatin (From Bovine Skin)
10. Denhardt's solution (refer to chapter V.C.2)

Procedure

a. Plate Coating With Avidin

1. Prepare a solution of avidin in Carbonate-Bicarbonate buffer. (10 mg of avidin in 100 ml of buffer).
2. Add 100 µl of avidin solution into each well of a 96-well microtiter plate.
3. Incubate the plates overnight at 4°C.
4. Next morning wash each well three times with 200 µl of Tris-Saline buffer.
5. After the third wash, block non-specific sites of the plates by adding 200 µl of Tris-Saline buffer containing 1% gelatin and 5 ml of Denhardt's solution. Incubate at 4°C.
6. Avidin coated plates can be stored like this for several weeks.

D. Conjugation of Oligonucleotide Probes to Horseradish Peroxidase

Purpose

Oligonucleotides that are directly conjugated to the enzyme horseradish peroxidase (HRP) can be used to detect DNA targets. This method of detecting hybridization of specific sequences has the advantages of less washing time, less hands-on time and, in most cases, a clear background. Disadvantages are that each probe has to be conjugated separately, consuming considerable time and resources in the process. Once conjugated, however, probes will last for a long time, as only a small volume of picomolar concentration is required for each assay.

In this procedure, a direct conjugation of HRP to oligonucleotide using a heterobifunctional cross linker is described. The general reaction scheme for this cross-linked coupling of HRP to an amino-oligonucleotide is shown in Figures 1, 2 and 3. The conjugation process involves a simple three-step method: 1) activation of HRP with crosslinker, 2) introduction of a sulfhydryl group to the amino-oligonucleotide, and 3) conjugation by mixing the activated HRP with chemically modified oligonucleotides. The second step can be avoided by synthesizing a thiol modified oligonucleotide, but it is found that yield is much higher using an amino modified oligonucleotide and introducing a sulfhydryl group chemically just before conjugation.

Instrumentation and Supplies

1. HPLC desalting set up
2. Oligonucleotide probes – All oligonucleotides listed in Table I used for conjugation are synthesized in 1 µM scale with amino dT C6 linker onto the 5'end via the phosphoramidite approach.
3. Peroxidase from Horseradish of RZ 3.0
4. Sulfo SMCC (Sulfosuccinimidyl 4-[N-maleimidomethyl]- cyclohexane-1-carboxylate)
5. SPDP (N- Succinimidyl 3-[2-pyridyldithio] propionate)
6. DTT (Dithiotheritol)
7. Phosphate-EDTA Buffer (200 mM Phosphate, 20mM EDTA, pH 7.5)
8. Desalting Buffer (20mM Phosphate, 2mM EDTA, pH 6.8)
9. PBS (Phosphate- Saline Buffer [100 mM phosphate, 150 mM Sodium Chloride, pH 6.8]

Procedure

a. Activation of HRP With Crosslinker

Dissolve 100 mg of HRP (2,500 moles) in 5 ml of Phosphate-EDTA Buffer. To this add 50 mg (11,500 moles) of Sulfo SMCC and incubate at room temperature for 60 minutes. After incubation, desalt the whole mixture on a desalting column using HPLC system. Calculate the HRP content in the yield and store lyophilized at –80°C until used for conjugation.

b. Introduction of Sulfhydryl Group to Amino Oligonucleotide

Dissolve 200 nM of amino oligonucleotide in 20 µl of Phosphate-EDTA buffer. To this add 40 µl of SPDP solution (100 mg SPDP dissolved in 3 ml of DMSO) and incubate at room temperature with occasional mixing. After 30 minutes, add 20 µl of DTT (77 mg dissolved in 1 ml water), incubate for a further 15 minutes at room temperature to reduce the disulfide bond, and desalt as above. Calculate the amount of oligonucleotide present and confirm the presence of a sulfhydryl group using Ellman's reagent.

c. Conjugation and Purification of HRP-Oligonucleotide

Dissolve 8 mg of lyophilized maleimide activated HRP in the sulfhydryl-containing solution of step b above and incubate in the dark at room temperature for at least 6 hours or overnight at 4°C. Finally add 50 mg of cysteine-HCl monohydrate and incubate at room temperature for an additional 60 min. Purify the conjugate on a Superdex-200 column using the HPLC system.

E. Procedure for Eldot Assay and Data Interpretation

Instrumentation

1. Shaking water baths
2. Micropipettors and tips
3. Microplate reader capable of reading at 450 nm
4. Microplate washer

Reagents and Supplies

1. 20X SSC (Sigma # S6639)
2. Hybridization buffer (1% Casein, 5X SSC, 0.5% SDS)
3. Amplicon Diluent (0.1M Tris-HCl, pH7.5, 100mM NaCl, 2mM EDTA)
4. 2X SSC (Prepared by diluting 20X SSC)
5. Denaturation Buffer (0.5M NaOH)
6. TMB Substrate Kit (Pierce 34021)
7. Stopping solution (1N Sulfuric Acid)
8. Tris-Saline buffer (0.1M Tris HCl, pH 7.5,150 mM NaCl)

Procedure

a. Sample Dilution

Dilute the PCR sample 1:20 in amplicon diluent. To obtain sufficient volume for ELDOT Assay, perform the PCR for HLA-DRB in duplicate and for HLA-A in triplicate. Dilute individual samples in tubes by adding 200 µl or 300 µl of amplicon and diluting it to 4 ml or 6 ml volume (1:20 dilution) by adding amplicon diluent buffer.

b. Assay Procedure

1. On the day of the assay, take the avidin coated trays out of 4°C and warm in a water bath for 15 minutes at 45°C, or enough for the gelatin to melt. Wash the tray using a micro titer plate washer 10 times with Tris-Saline buffer.
2. Add 100 µl of diluted PCR amplification product to corresponding well. Incubate at room temperature for 30 min to allow the biotinylated PCR products to bind to avidin coated trays.
3. Denature the DNA by adding 200 µl of denaturing buffer. Incubate for 10 min at room temperature.
4. Remove the non-biotinylated single strands of the DNA by washing 6 times with 2X SSC using a microtiter plate washer.
5. After the wash, hybridize the immobilized single stranded PCR product with 100 µl of sequence specific oligonucleotide probes conjugated with HRP (sequence shown in Tables 2 and 3). Dispense all probes with multichannel pipettor into the appropriate wells at working dilution (which should be predetermined by optimization studies) diluted with the hybridization buffer.
6. After one hour of hybridization at 45°C, discard the probes by inversion. Wash the plate six times with 2X SSC using a microtiter plate washer. Fill each well with 200 µl of 2X SSC and incubate the plate for 10 minutes at 45°C for HLA-A typings and 50°C for HLA-DRB typings. Repeat this procedure once more. Finally, rinse the plate five times with 2X SSC. Plates are now ready for detection.
7. Detect the binding of HRP conjugated probes to immobilized amplicon by adding 100 µl of TMB substrate. Prepare the substrate solution just ten minutes before use, according to manufacturer's instruction. Incubate at room temperature for 30 minutes. Stop the color development by adding 100 µl of stopping solution to each well, and read the absorbance at 450 nm using a micro titer plate reader.

c. Interpretation

To assign positive and negative cut-off optical density (OD) values, type at least 100 reference samples, which have been previously typed by dot blot hybridization procedure, by the ELDOT procedure. Include a well with no amplicon to be used as the negative control and the OD to be used as the background value.

In order to evaluate the results of hybridization independent of the efficiency of amplification obtained in separate PCR runs, use the monomorphic sequence SSOP of HLA-DRB alleles or HLA–A alleles (CX-1 for DRB or PS-10 for HLA-A) along with the polymorphic SSOP. Express the results as a ratio between OD reading of each SSOP and the OD reading of the monomorphic positive control. Calculate the mean values of the ratios of expected positive and reactions of each SSOP typing.

The best discrepancy rate is obtained when the cut-off is calculated from the mean value of the expected negative plus 2 standard deviations (SD). There should be no overlap between the values of the mean negative plus 2 SD with that of the lowest value of the expected positive sample. If any such value is obtained, the cut off point can be calculated as mean of expected negative plus 1 SD, or the oligonucleotide probes need to be changed. Compare the hybridization patterns of each unknown with that of expected for known alleles (Table 3 for HLA-A and Table 4 for HLA-DRB) to assign the oligo types for each sample. Although the cut off values for each probe should be determined empirically by each laboratory, an example of cut off values used by this laboratory is shown in Table 1 for HLA-A typing and in Table 2 for HLA-DRB typing.

Table : 1 Oligonucleotide Probes Used for HLA-A Typing

No	Probe Name	SEQUENCE	Suggested cut-off value in % of monomorphic positive.
1	RC-30	5′- GGTATTTCACCACATCCGTG-3′	22
2	RC-4	5′- CAGGAGAGGCCTGAGTAT-3′	24
3	PS-9	5′- ACGAGGAGACAGGGAAAG-3′	15
4	C-31	5′- TTGGGACCTGCAGACACGG-3′	32
5	PS-8	5′- CGGGGAGACACGGAAAGT-3′	18
6	RC-7	5′- CGGAACACACGGAATGTG-3′	31
7	RC-8	5′-AAGGCCCAGTCACAGACT-3′	46
8	RC-32	5′- GCCCACTCACAGACTGAC-3′	30
9	RC-33	5′- CCACTCACAGACTCACCG-3′	24
10	RC-9	5′- GAGTGGACCTGGGGACCC-3′	32
11	RC-10	5′- GAGCGAACCTGGGGACCC-3′	25
12	RC-43	5′- GACTGACCGAGAGAGCCTG-3′	45
13	RC-34	5′- CGGATCGCGCTCCGCTAC-3′	28
14	RC-12	5′- CACACCGTCCAGAGGATC-3′	31
15	RC-35	5′- CACACCATCCAGATAATG-3′	25
16	RC-13	5′- GGGTATGAACAGCACGCC-3′	10
17	RC-45	5′- TACCAGCAGGACGCTTACG-3′	35
18	RC-36	5′- CAGATCACCAAGCGCAAG-3′	47
19	RC-14	5′-GAGACGGCCCATGAGGCG-3′	31
20	RC-37	5′- GGCGGCCCATGTGGCG-3′	25
21	RC-38	5′- CGTCGGGCGGAGCAGTTG-3′	40
22	RC-39	5′- GGAGCAGTTGAGAGCC-3′	31
23	RC-15	5′- GGAGCAGTGGAGAGCC-3′	42
24	RC-16	5′- GAGCAGCAGAGAGCCTAC-3′	35
25	RC-17	5′- CTGGAGGGCCGGTGCGTG-3′	41
26	RC-40	5′-CTGGAGGGCGAGTGCGTG-3′	35
27	RC-18	5′- TGCGTGGACGGGCTCCGC-3′	30
28	RC-41	5′- TACCAGCAGGACGCCTACG-3′	45
29	RC-42	5′- AGGCGGCCCGTGTGGCGGA-3′	40
30	RC-44	5′- CTACCTGGATGGCACGTGC-3′	21
31	RC-22	5′- TGCGTGGAGTGGCTCCGC-3′	18
32	PS-11	5′- GCGGTCCATGCGGC-3′	22
33	RC-23	5′- CACAGACTAACCGAGCGA-3′	19
34	RC-24	5′- CAGACCACCAAGCACAAG-3′	24
35	RC-25	5′- CTGGAGGGCACGTGCGTG-3′	30
36	RC-46	5′- TGACCGAGAGAACCTG-3′	27
37	RC-48	5′- GGGTACCGGCAGGAC-3′	26
38	RC-49	5′- ACCTGGCGACCCTGCGCG-3′	25
39	PS-10	5′- CTGCGCTCTTGGACCGCG-3′	——

Table 2: Oligonucleotide Probes Used for HLA-DRB Typing

No	Probe Name	SEQUENCE	Suggested cut-off value in % of monomorphic positive.
1	CX-1	5′- CTGGAACAGCCAGAAGGAC-3′	——
2	CX-3	5′- TGGCAGCTTAAGTTTGAATGT-3′	17
3	CX-14	5′-TTGCAGCAGGATAAGTATGAG-3′	20
4	RC-113	5′-GGCCGIGTGGACAAITAC-3′	31
5	CX-7	5′-GAGCAGGTTAAACATGAGTGT-3′	29
6	NG-9	5′- CCTGATGAGGAGTACTGG-3′	12
7	RC-115	5′-AGGAGGIGCTCCTGCICTT-3′	20
8	RC-63	5′- GTACTCTACGTCTGAGTGTCA-3′	10
9	RC-88	5′- GAGTACTCTACGGGTGAG-3′	18
10	RC-145	5′- AGGCGGGICCTGGIGGAC-3′	15
11	CX-8	5′- TGGCAGGGTAAGTATAAGTGT-3′	17
12	RC-92	5′- CTGCGGTATCTGCACAGA-3′	12
13	RC-93	5′- GAAAGACGCGTCCATAACC-3′	12
14	RC-96	5′- ACATCCTGGAAGACGAGCG-3′	35
15	RC146	5′- GAGGAGTTCCTGCGCTT-3′	25
16	AS-6	5′- GAGCTGCTTAAGTCTGAG-3′	15
17	CX-4	5′- TGGCAGCCTAAGAGGGAGTGT-3′	10
18	RC-131	5′-TGGAGCIGAGGCIGGCC-3′	38
19	AS-10	5′- CTGCGGAGCACTGGAACA-3′	10
20	RC-128	5′- CTGGAAGACAAGCGGGCC-3′	35
21	RC-67	5′- ACCAGGAGGAGAACGTGC-3′	38
22	RC-129	5′- CTACGGIGCTITGGAGAG-3′	31
23	RC-148	5′- ITTCCTGGAGAGATACTTC-3′	25
24	RC-70	5′-CGGCCTAGCGCCGAGTAC-3′	27
25	RC-106	5′-GTTCCTGGAGAGACACTT-3′	18
26	RC-68	5′- CCAGGAGGAGTTCGTGC-3′	39
27	RC-149	5′-GGAGAAGAAGCIGGCCG-3′	21
28	RC-151	5′- GGTGCIGTACCTGGACA-3′	15
29	RC-72	5′- GACAGGCGGGCCGCGG-3′	44
30	RC-65	5′- GTTCCTGCACAGAGACAT-3′	15
31	RC-71	5′- GACCTCCTGGAAGACAGG-3′	20

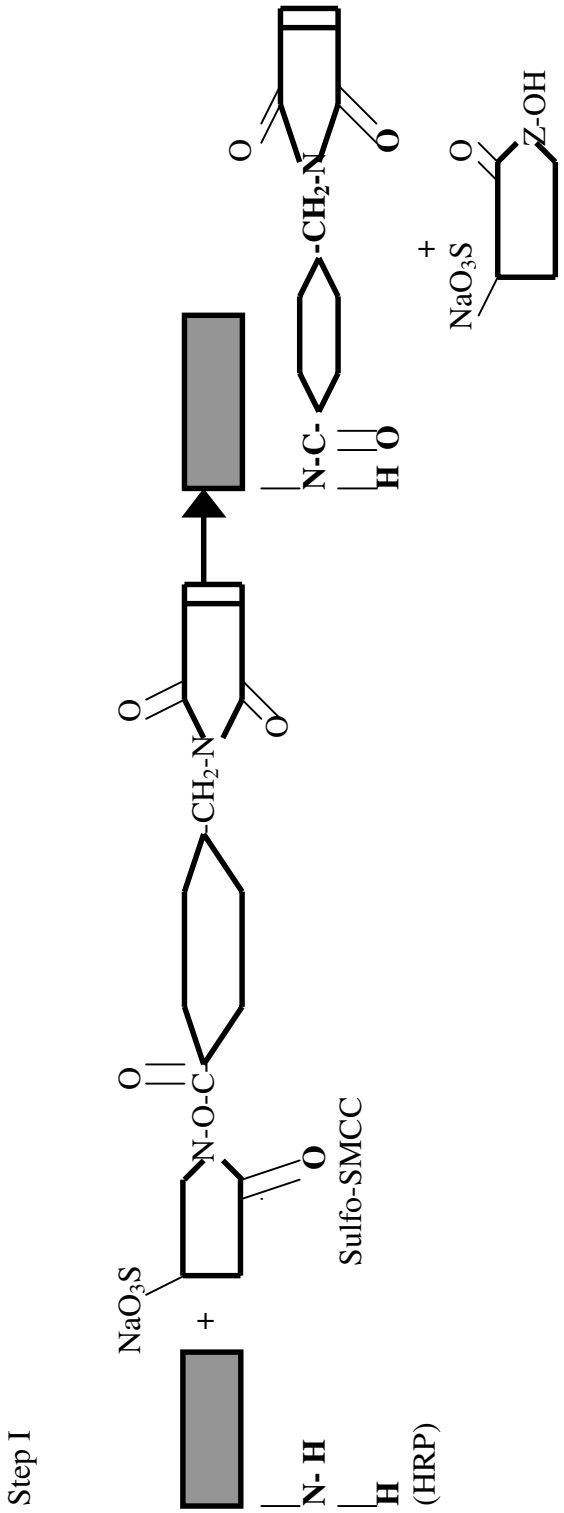
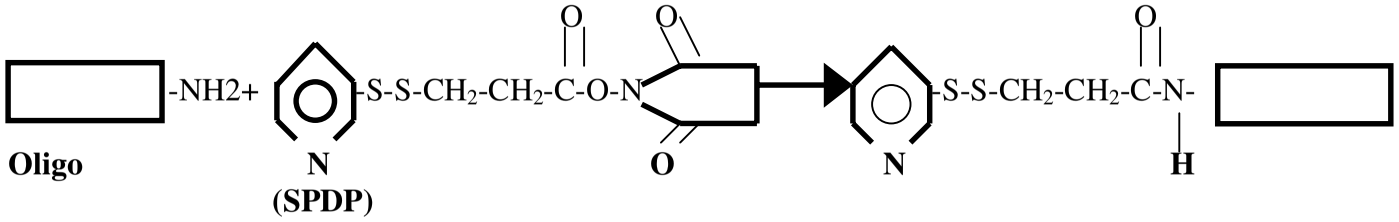


Figure 1- Activation of HRP

Step II

SPDP introduces a 2-pyridyl-disulfide when reacted with the primary amino oligo nucleotide.



To conjugate activiated HRP to the modified oligonucleotide, a free sulfhydryl is needed which can be obtained by reducing disulfide bonds using DTT.

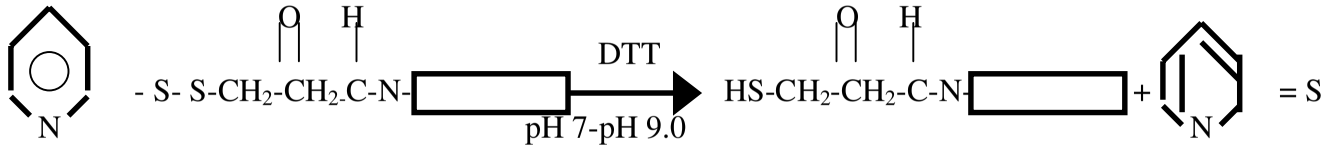


Figure 2- Introduction of sulfhydryl group to amino oligonucleotide

Step III- Conjugation

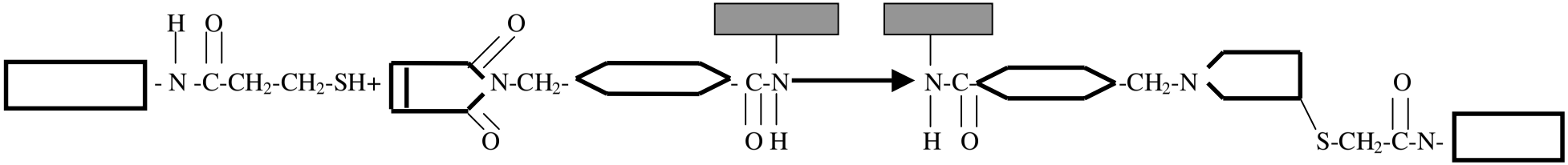


Figure 3: Final conjugation step.

Table 3 (Part I): HYBRIDIZATION FOR HLA-A ALLELES.

Allele List		0201	0202	0203	0204	0205	0206	0207	0208	0209	0210	0211	0212	0213	0214	0215n	0216	0217	0218	0219	0220	0221	0222	0224	0225	0226	680111	680112	6802	6803	6804	6805	2301	2402	2403	2404	2405	2406	2407	2408
#	Probes																																							
1	RC-30																																							
2	RC-4																																							
3	PS-9																															x	x	x	x	x	x	x	
4	RC-31																																							
5	PS-8	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x		x	x																	x
6	RC-7																										x	x	x	x	x	x								
7	RC-8																										x	x	x										x	
8	RC-32																													x			x	x	x	x	x	x		x
9	RC-33	x	x	x	x	x	x	x	x		x		x	x	x	x	x	x	x	x	x	x	x	x	x						x								
10	RC-9	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x							
11	RC-10																																x							
12	RC-43																																							
13	RC-34																															x	x	x		x	x	x	x	x
14	RC-12	x		x			x	x			x	x	x	x		x	x		x	x	x	x	x	x	x	x													
15	RC-35																																							
16	RC-13																																							
17	RC-45																																							
18	RC-36																			x														x	x	x		x	x	x
19	RC-14			x																																			
20	RC-37	x	x		x	x	x	x	x		x	x	x		x	x	x	x	x	x	x	x		x			x	x	x	x	x	x		x	x	x	x	x	x
21	RC-38																																							
22	RC-39	x			x		x	x							x	x	x	x	x		x	x	x	x	x	x							x						
23	RC-15		x	x		x			x														x				x	x	x	x	x	x					x		
24	RC-16												x	x						x														x	x	x	x		x	x
25	RC-17																																							
26	RC-40																x																							
27	RC-18																			x													x	x		x	x	x	x	x
28	RC-41																																							
29	RC-42																															x								
30	RC-44																																							
31	RC-22	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x		x	x	x	x	x	x	x	x	x	x	x			x					
32	PS-11																																							
33	RC-23																																							
34	RC-24	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x		x	x	x	x	x	x	x	x	x	x	x								
35	RC-25	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x		x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
36	RC-46																															x	x	x		x	x	x	x	
37	RC-48																										x	x		x	x	x								
38	RC-49																																							
39	PS-10	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x

Table 3 (Part II): HYBRIDIZATION FOR HLA-A ALLELES

#	Alleles	2409	2410	2413	2501	2502	2601	2602	2603	2604	2605	2606	2607	2608	2609	3401	3402	6601	6602	6603	2901	2902	2903	3001	3002	3003	3004	3101	3201	3202	3301	3303	7401	7402	7403	0101	0102	3601	0301	0302	0303	1101	1102	1103	1104	4301	8001
1	RC-30																				x	x	x					x			x	x														
2	RC-4																							x	x		x	x																			
3	PS-9	x	x	x																																												
4	RC-31																				x	x	x																								x	
5	PS-8												x	x																																		
6	RC-7				x	x	X	x	x	x	x	x		x	x		x	x	x	X											x	x															
7	RC-8					x										x	x	x	x		x	x	x	x																x	x	x	x	x	x	x			
8	RC-32	x	x	x	x		X	x		x	x		x	x	x					X					x	x	x		x	x			x	x	x	x	x	x							x	
9	RC-33								x			x																																			
10	RC-9								x			x				x	x	x	x	X				x				x			x	x	x	x					x	x	x	x	x	x	x		
11	RC-10						X	x		x			x	x	x						x	x	x															x	x	x						x	x
12	RC-43				x	x																							x	x																
13	RC-34	x	x	x	x	x																							x	x																
14	RC-12																																														
15	RC-35																x							x	x	x	x									x	x	x	x	x	x	x	x	x	x		x
16	RC-13																							x	x	x	x																			
17	RC-45				x	x	X		x	x	x		x	x	x	x		x	x	X																									x	
18	RC-36	x	x	x																																	x	x	x	x	x	x	x	x	x	x		x
19	RC-14				x	x	X	x	x	x	x	x	x	x	x	x	x	x	x	X																									x	
20	RC-37	x	x	x																							x			x											x					
21	RC-38																									x	x																			
22	RC-39			x												x					x	x	x	x	x	x		x	x		x	x	x	x	x				x		x					
23	RC-15				x	x	X	x	x	x	x	x	x		x	x		x	x	X							x																	x	
24	RC-16	x	x											x																x										x		x	x	x	x		
25	RC-17		x		x	x	X	x	x		x	x	x	x				x																			x	x				x	x	x		x	
26	RC-40																		x	X																										x
27	RC-18	x		x																			x														x	x								x
28	RC-41				x	x	X		x	x	x		x	x	x	x		x	x	X								x	x	x	x	x	x	x	x								x	
29	RC-42																				x	x	x					x	x		x	x	x	x	x										
30	RC-44																																						x	x	x						
31	RC-22		x		x	x	X	x	x	x	x	x	x	x	x	x	x	x	x	X	x	x		x	x	x	x	x	x	x	x	x	x	x	x			x	x	x	x	x	x	x	x	
32	PS-11																																			x	x	x								
33	RC-23																																														x
34	RC-24																																														
35	RC-25	x		x											x	x	x				x	x	x	x	x	x	x	x	x	x	x	x	x	x			x						x		
36	RC-46	x	x	x							x														x	x	x																			
37	RC-48																x				x	x	x													x	x	x	x	x	x	x	x	x		x
38	RC-49																																		x											
39	PS-10	x	x	x	x	x	X	x	x	x	x	x	x	x	x	x	x	x	x	X	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x

Table 4 Part I: HYBRIDIZATION FOR HLA-DRB ALLELES

Probes \ Alleles	0101	01021	01022	01032	0104	15011	15012	15021	15022	1503	1504	1505	1506	16011	16012	16021	16022	1603	1604	1605	1607	1608	03011	03012	03021	03022	03033	03034	03035	03055	03037	03038	03039	03111	04011	04012	04022	04033	04044	04051	04052	04066	04077	04088	04099	04100	04111	04122	04133	04144	04155	04166	04177	04188
1-CX-1	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
2-CX-3	x	x	x	x	x																																																			
3-CX-14																																																									
4-RC-113																							x		x		x	x	x	x	x	x	x	x																						
5-CX-7																																			x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
6-NG-9																																x																						x			
7-RC-115																																																									
8-RC-63																							x	x	x	x	x	x	x	x	x	x	x	x																							
9-RC-88																																																									
10-RC-145																			x																																									x
11-CX-8																																																									
12-RC-92																																																									
13-RC-93																																																									
14-RC-96				x																																	x																			x			
15-RC-146																																																									
16-AS-6																																																									
17-CX-4						x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x																																					
18-RC-131	x	x	x		x																																	x	x	x	x	x	x	x		x	x									x		
19-AS-10																																																								
20-RC-128																																																									
21-RC-67																						x	x	x	x	x	x		x	x	x	x		x																							
22-RC-129		x	x																																																						
23-RC-148																									x	x	x																														
24-RC-70																																										x	x				x	x	x	x					x		
25-RC-106																																																									
26-RC-68																																																									
27-RC149																																			x	x								x				x			x					
28-RC-151																							x	x				x	x	x		x	x	x																					
29-RC-72																	x																																							
30-RC-65																																																									
31-RC-71																x	x																																									

Table 4 Part II: HYBRIDIZATION FOR HLA-DRB ALLELES

Probes \ Alleles	0419	0420	0421	0422	0423	0424	1101	1102	1103	1102	1103	1104	1104	1105	1106	1107	1108	1108	1109	1110	1111	1112	1113	1114	1115	1116	1117	1118	1119	1120	1121	1122	1123	1124	1125	1126	1127	1128	1129	1130	1201	1202	1202	1203	1203	1204	1205	1301	1302	1303	1303	1304	1305	1306	1307
1-CX-1	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
2-CX-3																																																									
3-CX-14																																																									
4-RC-113				x												x																																											
5-CX-7	x	x	x	x	x	x																									x																											
6-NG-9							x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x		x	x	x	x	x	x	x	x				x																
7-RC-115																																								x	x	x	x	x	x															
8-RC-63							x	x	x	x	x	x	x		x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x		x	x	x	x	x	x	x	x							x	x	x	x	x	x	x	x	x	x	x	x
9-RC-88																																							x	x	x	x	x	x	x														
10-RC-145																															x		x																										
11-CX-8																																																												
12-RC-92																																																											
13-RC-93																																																											
14-RC-96										x														x			x																						x	x			x						
15-RC-146																																																											
16-AS-6																																																											
17-CX-4																																																											
18-RC-131	x	x			x																															x																							
19-AS-10																																																											
20-RC-128																																																									x	x				
21-RC-67																			x							x				x																								x	x			x	x	
22-RC-129															x																x										x	x	x				x	x									
23-RC-148																																																											
24-RC-70						x																																																					
25-RC-106																																																											
26-RC-68																				x			x				x																																
27-RC149			x																																																									
28-RC-151																																																											
29-RC-72							x	x	x			x	x	x	x		x	x	x	x		x			x			x	x			x		x			x	x	x	x				x									x	x	x
30-RC-65																																																										
31-RC-71																	x	x																																									

Table 4 Part III: HYBRIDIZATION FOR HLA-DRB ALLELES

Alleles / Probes	1308	1309	1310	1311	1312	1313	1314	1315	1316	1317	1318	1319	1320	1321	1322	1323	1324	1325	1326	1327	1328	1329	1330	1401	1402	1403	1404	1405	1406	1407	1408	1409	1410	1411	1412	1413	1414	1415	1416	1417	1418	1419	1420	1421	1422	1423	1424	1425	1426	1427	1428	1429	0701
1-CX-1	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	X	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
2-CX-3																																																						
3-CX-14																																																						
4-RC-113																																																						
5-CX-7																																	x																					
6-NG-9																																		x																			
7-RC-115																																																					
8-RC-63	x	x	x	x	x	x	x	x	x		x	x	x	x	x	x	x	X	x	x	x	x	x	x	x	x		x	x	x	x	x			x	x	x		x	x	x	x	x	x	x	x	x	x	x		x	
9-RC-88										x																	x							x					x														
10-RC-145						x					x															x								x				x											x				
11-CX-8																																																					x
12-RC-92																																																					
13-RC-93																																																					
14-RC-96	x							x	x	x		x			x	x				x	x		x															x																
15-RC-146																																																					
16-AS-6																																																					
17-CX-4																																																					
18-RC-131																									x				x			x				x					x			x							x	
19-AS-10																								x			x			x																	x			x	x		x		
20-RC-128			x																																																			
21-RC-67		x	x					x	x		x		x						x	x	x	x			x	x			x			x				x	x				x	x	x		x			x		x		x	
22-RC-129																																																				x	x	
23-RC-148								x				x							x						x	x			x						x	x				x	x	x				x					x	
24-RC-70					x	x								x									x													x																	
25-RC-106																																																					
26-RC-68	x											x												x			x	x		x	x		x	x				x	x	x				x		x	x			x		x	
27-RC149																																																					
28-RC-151																				x																																		
29-RC-72				x	x		x							x				X					x																															
30-RC-65																																																					
31-RC-71													x					X								x									x																	

Table 4 Part IV: HYBRIDIZATION FOR HLA-DRB ALLELES

Alleles / Probes	0801	0802 1	0802 2	0803 1	0803 2	0804 1	0804 2	0805	0806	0807	0808	0809	0810	0811	0812	0813	0814	0815	0816	0901 1	0901 2	1001	B3 0101	B3 0102	B3 0201	B3 0202	B3 0203	B3 0204	B3 0205	B3 0206	B3 0301	B4 0101 1	B4 0102	B4 0103	B4 0104	B4 0105	B4 0201	B5 0101	B5 0101 2	B5 0102	B5 0103	B5 0104	B5 0105	B5 0201	B5 0202	B5 0203	B5 0204
1-CX-1	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	X	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
2-CX-3																																															
3-CX-14																																						x	x	x	x	x	x	x	x	x	x
4-RC-113																												x																			
5-CX-7																																															
6-NG-9																																															
7-RC-115																																															
8-RC-63																																															
9-RC-88	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x		x	x																												
10-RC-145	x	x	x	x	x	x	x		x	x	x	x	x	x	x	x	x	x	x																												
11-CX-8																																															
12-RC-92																				X	x																										
13-RC-93																						x																								
14-RC-96																																															
15-RC-146																							x	x																						
16-AS-6																									x	x	x	x	x	x	x															
17-CX-4																																															
18-RC-131																																															
19-AS-10										x																																					
20-RC-128																																															
21-RC-67																																															
22-RC-129															x																													x	x		x
23-RC-148																													x		x																
24-RC-70	x			x	x			x	x				x		x		x		x																												
25-RC-106																								x	x	x	x		x																
26-RC-68												x																			x																
27-RC149																																															
28-RC-151																							x	x																						
29-RC-72								x																															x								
30-RC-65																																						x	x			x	x				
31-RC-71																x																														

References

1. Ahn, J.S., Costa, J. and Emanuel, R.J. Pico green quantitation of DNA: effective evaluation of samples pre- or post –PCR. Nucleic Acid Res. 24: 2623, 1996.
2. Bhatia, S.K., Shriver- Lake, L.C., Prior, K.J., Georger, J.H., Calvert, J.M., Brodehost, R. and Ligler, F.C. Use of thiol terminal silanes and hetrobifunctional crosslinkers for immobilization of antibodies on silica surfaces. Anal. Biochem. 178:408, 1989
3. Bos, E.E. 3,3'5, 5'- Tetramethylbenzidine as an Ames test negative chromogen for horseradish peroxidase in enzyme immunoassay. J. Immunoassay, 2: 187, 1981.
4. Bugawan, T. L., Apple, R. and Erlich, H. A method for typing polymorphism at the HLA-A locus using PCR amplification and immobilized oligonucleotide probes. Tissue Antigens. 44 : 137, 1994.
5. Bunce, M., O'Neill, C., Banardo, C. Phototyping: Comprehensive DNA typing for HLA- A, B, C, DRB1, DRB3, DRB4, DRB5, and DQB1 by PCR with 144 primer mixes utilizing sequence- specific primers (PCR-SSP). Tissue Antigens. 46: 355, 1995.
6. Fernandez- Vina, M. Shumway, W., Stastney, P. DNA typing for class II HLA antigens with allele specific or group specific amplification. II Typing for alleles of the DRw 52 – associated group. Human Immunol. 27: 51, 1990.
7. Goodchild, J. Conjugates of oligonucleotides and modified oligonucleotides: a review of their synthesis and properties. Bioconjugate Chem. 1: 165, 1990.
8. Hashida, S., Imagawa, M., Inque, S., Ruan, K.H. and Ishikawa, E. More useful melamine compounds for the conjugation of Fab to horseradish peroxidase through thiol group in the hinge. J. Appl. Biochem. 6:56,1984.
9. Lazaro , A.M., Fernandez- Vina M.A., Liu Z., Stastny, P. Enzyme- linked oligotyping (ELDOT): A practical method for clinical HLA-DNA typing. Human Immunol. 36: 243, 1993.
10. Leary, J.J., and Ruth, J.L. Nonradioactive labelling of nucleic acid probes in Nucleic Acid and Monoclonal Antibody Probes: Applications in Diagnostic Microbiology. (Swaminathan, B. and Prakash, G. Eds.) Marcel Dekker, New York, pp33-57, 1989.
11. Liem, H., Cardenas, F., Tavassoli, M., Poh-Fitzpatric, M. and Muller- Eberhard, U. Quantitative determination of hemoglobin and cytochemical staining for peroxides using 3,3'5, 5'- tetramethyl –benzidine dihydrochloride, a safe substitute for Benzidine. Anal. Biochem. 98: 388, 1979.
12. Scharf, S.J., Griffith, R.L., and Erlich, H. Rapid typing of DNA sequence polymorphism at the HLA-DRB1 locus using the polymerase chain reaction and nonradioactive oligonucleotide probes. Human Immunol. 30: 190, 1991.
13. Sheldon, E.L., Kellogg, D.E., Watson, R., Levenson, C. and Erlich, H. Use of nonisotopic M13 probes for genetic analysis: application to class II loci. Proc. Natl. Acad. Sci. USA. 83: 9085,1986 .
14. Yoshitake, S. Mild and efficient conjugation of rabbit Fab and horseradish peroxidase using a maleimide compound and its use for enzyme immunoassay. J. Biochem. 92:1413, 1982.

Commercial Vendors of Kits for Molecular Typing

Brian F. Duffy

Purpose

Nucleic acid based methods for HLA typing are quick, accurate, and competitively priced compared to serological methods. The popularity of DNA based typing methods attracted the attention of commercial vendors, who now offer many choices of formats. Since most of the optimization of primer and probe design and master mix titration has already been performed, and analysis software is easy to interpret, PCR-SSP and PCR-SSOP are probably the best methods to employ in laboratories new to HLA DNA typing methods. The different methods allow laboratories to choose based on available equipment (ELISA readers, electrophoresis apparatus and thermalcyclers). This is a list of vendors that compares PCR-SSP and PCR-SSOP products, to aid in the decision of which format is best for laboratories initiating HLA DNA typing, and to serve as an update for laboratories already performing nucleic acid based typing.

Specimens

The PCR reaction requires template DNA extracted from nucleated cells. Some vendors supply an extraction reagent with the price of the kit. These are designated “yes” in Ex Reg column, while vendors that provide kits at an extra charge are designated “avail”. When choosing a DNA extraction process for any PCR testing, consult the vendor’s suggestions for acceptable methods and anticoagulants.

Reagents and Supplies

Most of the kits listed include all of the components required for DNA amplification and detection with the exception of Taq polymerase. Kits that include Taq polymerase in the price of the kit are designated “yes” in the “taq” column.

Instrumentation

A thermalcycler is required for PCR, and most vendors provide suggested amplification conditions. SSP requires electrophoresis and documentation equipment such as a camera or digital scanner. SSOP requires waterbaths or chambers for hybridization and washing, as well as a means of detection, usually colorimetric or chemiluminescent.

Calibration

Not Applicable

Quality Control

The SSP and SSOP techniques employ the polymerase chain reaction. PCR is covered by United States patents 4,683,195 and 4,683,202. The use of the PCR reaction for *in vitro* diagnostic procedures requires either the laboratory or its institution to purchase a license from F. Hoffman LaRoche Ltd. or Roche Molecular Systems Inc. This license typically requires an up-front fee and a percentage of revenues. Another alternative is to purchase a product that carries the PCR license. In this instance, the PCR license was purchased by the vendor and is included in the price of the kit. These kits are designated with “yes” in the “PCR” column.

Laboratories that intend to use any commercial kit as the sole indicator of an *in vitro* diagnostic procedure must use a product submitted by the vendor and approved by the FDA for *in vitro* diagnostic use. These products are indicated as “IVD” in the “FDA” column. Kits not cleared for *in vitro* use are designated “RUO”. Analyte Specific Reagent (ASR) is new FDA classification of reagent that applies to manufacturers that market “home brew” tests without obtaining specific FDA clearance for that reagent. Although all of these kits are tested by the vendor, ASHI standards require laboratories demonstrate competence in using all commercial kits, including kits approved for *in vitro* diagnostic use. This means validating the performance of as many of the kit’s primer pairs as possible before placing the product in service. This data must be available for review by accrediting agencies.

Procedure

Not Applicable

Calculations

Not Applicable

RESULTS:

								SSP				
Company	Product Name	PCR	FDA	DNA Ex	taq	alqt	°C	HLA Loci/allele group	# primer sets	resolution	typings/ kit	Cost/ Typing
Biotest	HLA-A SSP	Yes	RUO	no	no	yes	4	HLA-A*01 -A*80	24	22/88	12	$26.00
	HLA-B SSP	Yes	RUO	no	no	yes	4	HLA-*07-B*82	48	49/82	12	$37.00
	HLA-C SSP	Yes	RUO	no	no	yes	4	HLA Cw*01-*1802	16	16/45	12	$23.50
	DRB SSP	Yes	RUO	no	no	yes	-20	DR1-18, DR51,DR52,DR53	22	21/21	12	$28.75
	DR4 SSP	Yes	RUO	no	no	yes	-20	DRB1*0401- DRB1*0422	13	22/22	12	$21.25
	DQB1 & DQA1 SSP	Yes	RUO	no	no	yes	-20	DQA1*0101-DQA1*0601 DQB1*05-DQB1*04	22	DQA1 10/10 DQB1 7/7	12	$28.75
Biosynthesis	Fastype HLA-A low resolution	no	RUO	avail	no	yes	-20	HLA A*1-A*80	24	22/88	25	$22.00
	Fastype HLA-B low resolution	no	RUO	avail	no	yes	-20	HLA B*7-B*82	46	48/81	25	$46.00
	Fastype HLA-Cw inter. resolution	no	RUO	avail	no	yes	-20	HLA Cw*0101-*1802	23	10/45	25	$22.00
	Fastype DR low resolution	no	RUO	avail	no	yes	-20	DR1-18 , DR51,DR52,DR53	24	39/130	25	$21.00
	Fastype DRB1*01 high resolution	no	RUO	avail	no	yes	-20	DRB1*0101-*0104	6	5/5	25	$8.00
	Fastype DRB1*15 high resolution	no	RUO	avail	no	yes	-20	DRB1*1501-*1506	8	8/8	25	$8.00
	Fastype DRB1*16 high resolution	no	RUO	avail	no	yes	-20	DRB1*1601-*1608	8	9/9	25	$8.00
	Fastype DRB1*03 high resolution	no	RUO	avail	no	yes	-20	DRB1*0301-*0311	14	7/7	25	$8.00
	Fastype DRB1*04 high resolution	no	RUO	avail	no	yes	-20	DRB1*0401-*0422	16	22/22	25	$16.00
	Fastype DRB1*11 high resolution	no	RUO	avail	no	yes	-20	DRB1*1101-*1131	16	22/22	25	$15.20
	Fastype DRB1*12 high resolution	no	RUO	avail	no	yes	-20	DRB1*1201-*1205	8	3/3	25	$6.00
	Fastype DRB1*13 high resolution	no	RUO	avail	no	yes	-20	DRB1*1301-*1333	16	22/22	25	$8.00
	Fastype DRB1*14 high resolution	no	RUO	avail	no	yes	-20	DRB1*1401-*1431	16	20/20	25	$8.00
	Fastype DRB1*08 high resolution	no	RUO	avail	no	yes	-20	DRB1*0801-*0819	16	11/11	25	$14.00
	Fastype DRB3* High resolution	no	RUO	avail	no	yes	-20	DRB3*0101-0303	13	19/19	25	$6.00
Biosynthesis	Fastype DRB4* High resolution	no	RUO	avail	no	yes	-20	DRB4*0101-0201N	8	7/7	25	$6.00
	Fastype DRB3/4/5 High resolution	no	RUO	avail	no	yes	-20	DRB5*0101-*0204	13	13/13	25	$8.00

Company	Product Name	PCR	FDA	DNA Ex	taq	alqt	°C	HLA Loci/allele group	primer sets	resolution	typings/ kit	cost/ typing
	Fastype DQ low resolution	no	RUO	avail	no	yes	-20	DQB1*02,*03,*04,*05-*09	8	11/11	25	$14.00
	Fastype DQ high resolution	no	RUO	avail	no	yes	-20	DQB1*0201-*06	24	30/33	25	$20.00
	Fastype DR/ DQ low resolution	no	RUO	avail	no	yes	-20	DR 1-18, DR51,52,53 DQB1*02,03,04,05-09	32		25	$25.00
Dynal	AllSet SSP HLA-A low resolution	no	RUO	avail	no	yes	-20	HLA A*01-A*80	24	21/21	20	$26.25
	AllSet SSP HLA-B low resolution	no	RUO	avail	no	yes	-20	HLA B*07-B*81	48	35/35	20	$42.50
	AllSet SSP HLA-C low resolution	no	RUO	avail	no	yes	-20	HLA Cw*01-*18	18	15/15	20	$19.25
	AllSet SSP DR low resolution	no	RUO	avail	no	yes	-20	DR1-18, DR51,DR52,DR53	24	14/14	20	$25.75
	AllSet SSP DQ low resolution	no	RUO	avail	no	yes	-20	DQB1*02-*04	8	11/11	20	$14.25
Dynal	HLA-A low resolution SSP	no	RUO	avail	no	no	-20	HLA-A*0101-*8001	24	0101-8001	50	$25.70
	HLA-A1, -A3, -A11, -A68	no	RUO	avail	no	no	-20	HLA-A1,A3,A11,A68	14	14/14	25	$14.80
	HLA-A2	no	RUO	avail	no	no	-20	HLA-A*0201-A*0223	24	23/23	25	$25.80
	HLA-A9	no	RUO	avail	no	no	-20	HLA-A*2301-A*2414	12	14/14	25	$15.60
	HLA-10	no	RUO	avail	no	no	-20	HLA-10	25	17/17	25	$19.00
	HLA-B16	no	RUO	avail	no	no	-20	HLA-B*3801-*3802,*3901-*3912	16	17/17	25	$20.00
	HLA-B17	no	RUO	avail	no	no	-20	HLA-B*5701-*5704, *5801-*5802	8	6/6	25	$13.40
	HLA-B18	no	RUO	avail	no	no	-20	HLA-B*1801-*1805	6	5/5	25	$13.40
	HLA-B22	no	RUO	avail	no	no	-20	HLA-B*5401,*5501-*5703	12	4/4	25	$19.20
	HLA-B27	no	RUO	avail	no	no	-20	HLA-B*2701-*2711	12	12/12	25	$16.60
	HLA-B35	no	RUO	avail	no	no	-20	HLA-B*3501-*3521	16	22/22	25	$20.00
	HLA-B40	no	RUO	avail	no	no	-20	HLA-B*4001-*4011	12	12/12	25	$19.20
	HLA-B27 high resolution	no	RUO	avail	no	no	-20	HLA-B*2701-B*2711	12	11/11	25	$16.60
	HLA-Cw low resolution SSP	no	RUO	avail	no	no	-20	HLA-Cw* 0102-*1802	18	36/36	25	$18.40
	HLA-Cw*07	no	RUO	avail	no	no	-20	HLA-Cw* 0701-*0707	25	7/7	25	$12.60
Dynal	DR low resolution SSP	no	RUO	avail	no	no	-20	DR1-18,103 ,DR51,DR52,DR53	24	17/17	50	$24.50

Company	Product Name	PCR	FDA	DNA Ex	tag	alqt	°C	HLA Loci/allele group	primer sets	resolution	typings/ kit	cost/ typing
	DRB1*01	no	RUO	avail	no	no	-20	DRB1*0101-*0104	5	5/5	25	$10.00
	DRB1*02	no	RUO	avail	no	no	-20	DRB1*1501-*1506,*1601-*08	12	18/18	25	$16.60
	DRB1*03	no	RUO	avail	no	no	-20	DRB1*0301-*0311	8	13/13	25	$10.60
	DRB1*04	no	RUO	avail	no	no	-20	DRB1*0401-*0427	16	29/29	25	$18.40
	DRB1*11	no	RUO	avail	no	no	-20	DRB1*11011-*1131	16	35/35	25	$16.60
	DRB1*08/12	no	RUO	avail	no	no	-20	DRB1*0801-*0817, *1201-*1205	12	28/28	25	$16.60
	DRB1*13	no	RUO	avail	no	no	-20	DRB1*1301-*1333	16	34/34	25	$18.40
	DRB1*14	no	RUO	avail	no	no	-20	DRB1*1401-*1430	16	30/30	25	$16.60
	DRB3*	no	RUO	avail	no	no	-20	DRB3*01011-*0103,*0201-*0207,*0301	12	15/15	50	$22.20
	DRB4*	no	RUO	avail	no	no	-20	DRB4*0101101-*0301N	8	5/5	50	$9.20
	DRB5*	no	RUO	avail	no	no	-20	DRB5*0101-*0109, *0202-*0204	12	12/12	50	$12.90
	DQ low resolution	no	RUO	avail	no	no	-20	DQB1*02,*03,*04,*05,*06	8	5/5	50	$13.60
	DQB1*05	no	RUO	avail	no	no	-20	DQB1*0501-*0504	4	5/5	25	$9.60
	DQB1*06	no	RUO	avail	no	no	-20	DQB1*0601-*0613	12	15/15	25	$19.20
	DQB1*02/04	no	RUO	avail	no	no	-20	DQB1*0201-*0203, *0401-*0402	6	5/5	25	$11.20
	DQB1*03	no	RUO	avail	no	no	-20	DQB1*0301-*0307	8	8/8	25	$12.60
	DQA1	no	RUO	avail	no	no	-20	DQA1*0101-*0601	20	18/18	50	$18.20
	DPB1	no	RUO	avail	no	no	-20	DPB1*01-*79	48	75/75	50	$40.50
	DPA1	no	RUO	avail	no	no	-20	DPA1*01*-04	8	4/4	25	$14.80
GenoVision	A-B-DR combi tray	yes	RUO	no	yes	yes	4/-20	HLA A,B,DR	96	serological level	24	$101.00
	A-B-C combi tray	yes	RUO	no	yes	yes	4/-20	HLA A,B,C	92	serological level	24	$97.00
	DR-DQ combi tray	yes	RUO	no	yes	yes	4/-20	HLA DR,DQ	32	serological level	48	$35.00
GenoVison	HLA-A "low"	yes	RUO	no	yes	yes	4/-20	HLA A	24	serological level	48	$28.00
	HLA-A "low" bulk	yes	RUO	no	yes	no	4/-20	HLA A	24	serological level	24	$28.00
	HLA-A1	yes	RUO	no	yes	yes	4/-20	HLA A1	5	allelic	24	$8.00
	HLA-A2	yes	RUO	no	yes	yes	4/-20	HLA A2	24	allelic	24	$28.00
	HLA-A3	yes	RUO	no	yes	yes	4/-20	HLA A3	4	allelic	24	$7.00

Company	Product Name	PCR	FDA	DNA Ex	tag	alqt	°C	HLA Loci/allele group	primer sets	resolution	typings/ kit	cost/ typing
	HLA-A9	yes	RUO	no	yes	yes	4/-20	HLA A9	12	allelic	24	$21.00
	HLA-A10	yes	RUO	no	yes	yes	4/-20	HLA-A10	16	allelic	12	$27.00
	HLA-A11	yes	RUO	no	yes	yes	4/-20	HLA A11	6	allelic	12	$11.00
	HLA-A19	yes	RUO	no	yes	yes	4/-20	HLA A19	16	allelic	24	$26.00
	HLA-A68	yes	RUO	no	yes	yes	4/-20	HLA 68	8	allelic	12	$13.50
GenoVision	HLA-B "low"	yes	RUO	no	yes	yes	4/-20	HLA B	48	allelic	48	$53.00
	HLA-B "low" bulk	yes	RUO	no	yes	no	4/-20	HLA B	48	allelic	24	$53.00
	HLA-B5	yes	RUO	no	yes	yes	4/-20	HLA B5	16	allelic	12	$27.00
	HLA-B7	yes	RUO	no	yes	yes	4/-20	HLA B7	12	allelic	24	$21.00
	HLA-B8	yes	RUO	no	yes	yes	4/-20	HLA B8	8	allelic	24	$13.00
	HLA-B12	yes	RUO	no	yes	yes	4/-20	HLA B12	12	allelic	24	$21.00
	HLA-B13	yes	RUO	no	yes	yes	4/-20	HLA B13	5	allelic	12	$9.00
	HLA-B14	yes	RUO	no	yes	yes	4/-20	HLA B14	6	allelic	12	$8.00
	HLA-B15	yes	RUO	no	yes	yes	4/-20	HLA B15	32	allelic	12	$52.00
	HLA-B16	yes	RUO	no	yes	yes	4/-20	HLA B16	16	allelic	24	$27.00
	HLA-B17	yes	RUO	no	yes	yes	4/-20	HLA B17	8	allelic	12	$13.50
	HLA-B18	yes	RUO	no	yes	yes	4/-20	HLA B18	8	allelic	12	$13.50
	HLA-B22	yes	RUO	no	yes	yes	4/-20	HLA B22	12	allelic	12	$21.00
	HLA-B27 low resolution	yes	RUO	no	yes	yes	4/-20	HLA B27	1	serological level	72	$7.00
	HLA-B27 bulk	yes	RUO	no	yes	no	4/-20	HLA B27	1	serological level	48	$7.00
	HLA-B27 high resolution	yes	RUO	no	yes	yes	4/-20	HLA B*2701-*2713	12	allelic	24	$21.00
	HLA-B35	yes	RUO	no	yes	yes	4/-20	HLA B35	16	serological level	24	$24.00
GenoVision	HLA-B40	yes	RUO	no	yes	yes	4/-20	HLA B40	16	serological level	24	$24.00
	HLA-C "low"	yes	RUO	no	yes	yes	4/-20	HLA C	20	serological level	24	$23.00
	HLA-C "low bulk"	yes	RUO	no	yes	no	4/-20	HLA C	20	serological level	24	$23.00
	HLA-Cw*03	yes	RUO	no	yes	yes	4/-20	HLA Cw 03	8	allelic	12	$13.00
	HLA-Cw*04	yes	RUO	no	yes	yes	4/-20	HLA Cw 04	6	allelic	12	$8.00
	HLA-Cw*05	yes	RUO	no	yes	yes	4/-20	HLA Cw 05	3	allelic	12	$5.00

Company	Product Name	PCR	FDA	DNA Ex	tag	alqt	°C	HLA Loci/allele group	primer sets	resolution	typings/ kit	cost/ typing
	HLA-Cw*06	yes	RUO	no	yes	yes	4/-20	HLA Cw 06	4	allelic	12	$7.00
	HLA-Cw*07	yes	RUO	no	yes	yes	4/-20	HLA Cw 07	12	allelic	24	$21.00
	DR "low"	yes	RUO	no	yes	yes	4/-20	DR 1-18 DR51,52,53	24	serological level	48	$27.00
	DR "low" bulk	yes	RUO	no	yes	no	4/-20	DR 1-18 DR51,52,53	24	serological level	24	$27.00
	DRB1*01	yes	RUO	no	yes	yes	4/-20	DRB1*0101-*0104	5	allelic	24	$8.00
	DRB1*15/16	yes	RUO	no	yes	yes	4/-20	DRB1*1501-*1506, *1601-*1608	16	allelic	24	$24.00
	DRB1*03	yes	RUO	no	yes	yes	4/-20	DRB1*0301-*0311	8	allelic	24	$12.00
	DRB1*04	yes	RUO	no	yes	yes	4/-20	DRB1*0401-*0429	16	allelic	24	$24.00
	DRB1*11	yes	RUO	no	yes	yes	4/-20	DRB1*1101-*1132	16	allelic	24	$24.00
	DRB1*13	yes	RUO	no	yes	yes	4/-20	DRB1*1301-*1333	16	allelic	24	$24.00
	DRB1*14	yes	RUO	no	yes	yes	4/-20	DRB1*1401-*1431	16	allelic	12	$26.00
	DRB1*07	yes	RUO	no	yes	yes	4/-20	DRB1*0701-*0704	4	allelic	24	$6.00
	DRB1*08/12	yes	RUO	no	yes	yes	4/-20	DRB1*0801-*0819, *1201-*1205	16	allelic	12	$26.00
	DRB3	yes	RUO	no	yes	yes	4/-20	DRB3*0101-*0104, *0201-*0208, *0301-*0303	16	allelic	24	$24.00
	DRB4	yes	RUO	no	yes	yes	4/-20	DRB4*0101-*0301N	8	allelic	24	$12.00
	DRB5	yes	RUO	no	yes	yes	4/-20	DRB5*0101-*0109, *0202-0204	12	allelic	24	$19.00
	DRA1	yes	RUO	no	yes	yes	4/-20	DRA1*0101-*0102	2	allelic	24	$3.50
GenoVision	DQ "low"	yes	RUO	no	yes	yes	4/-20	HLA DQ	8	serological level	48	$9.50
	DQ "low" bulk	yes	RUO	no	yes	no	4/-20	HLA DQ	8	serological level	24	$9.50
	DQB1*05	yes	RUO	no	yes	yes	4/-20	DQB1*0501-*0504	5	allelic	24	$8.00
	DQB1*06	yes	RUO	no	yes	yes	4/-20	DQB1*0601-*0614	15	allelic	24	$23.00
	DQB1*02	yes	RUO	no	yes	yes	4/-20	DQB1*0201-*0203	4	allelic	24	$6.00
	DQB1*03	yes	RUO	no	yes	yes	4/-20	DQB1*0301-*0308	8	allelic	24	$12.00
	DQB1*04	yes	RUO	no	yes	yes	4/-20	DQB1*0401-*0402	3	allelic	12	$5.00
	DQA1	yes	RUO	no	yes	yes	4/-20	DQA1*0101-*0601	20	allelic	24	$26.00
	DPA1	yes	RUO	no	yes	yes	4/-20	DPA1*0103-*0401	12	allelic	24	$19.00

Company	Product Name	PCR	FDA	DNA Ex	taq	alqt	°C	HLA Loci/allele group	primer sets	resolution	typings/ kit	cost/ typing
	DPB1	yes	RUO	no	yes	yes	4/-20	DPB1*0101-*7901	48	allelic	24	$70.00
GenoVision	all products are also available w/o PCR license for 25% less list price											
GenYsys/ GenTrak	HLA-A Low Res	no	RUO	avail	no	yes	-20		24		10	
	HLA-B Low Res	no	RUO	avail	no	yes	-20		46		10	
	HLA-C Intermediate Res	no	RUO	avail	no	yes	-20		20		10	
	DRB1* Low Res	no	RUO	avail	no	yes	-20		24		10	
	DQB1* Low Res	no	RUO	avail	no	yes	-20		8		10	
	DQB1* High Res	no	RUO	avail	no	yes	-20		24		10	
	DRB1* 01 High Res	no	RUO	avail	no	yes	-20		6		10	
	DRB1* 15 High Res	no	RUO	avail	no	yes	-20		6		10	
	DRB1* 16 High Res	no	RUO	avail	no	yes	-20		24		10	
	DRB1* 03 High Res	no	RUO	avail	no	yes	-20		6		10	
	DRB1* 04 High Res	no	RUO	avail	no	yes	-20		16		10	
	DRB1* 11 High Res	no	RUO	avail	no	yes	-20		15		10	
	DRB1* 12 High Res	no	RUO	avail	no	yes	-20		4		10	
	DRB1* 13 High Res	no	RUO	avail	no	yes	-20		20		10	
	DRB1* 14 High Res	no	RUO	avail	no	yes	-20		20		10	
One Lambda	Micro SSP HLA-ABC Generic	yes	IVD	no	yes	yes	-20	A,B,C	96	serological level	10	$92.00
	Micro SSP LMT Supplement	yes	IVD	no	yes	yes	-20	A,B	24	serological level	12	$30.67
	Micro SSP Seramates (A Locus)	yes	IVD	no	yes	yes	-20	A26,31,33,36,68,69,74	8	serological level	48	$7.67
	Micro SSP Seramates (B15)	yes	IVD	no	yes	yes	-20	B62,63,71(70),72(70),75,76,77	16	serological level	24	$15.33
	Micro SSP Seramates (B35,53)	yes	IVD	no	yes	yes	-20	B35,53	8	serological level	48	$7.67
	Micro SSP Seramates (B40)	yes	IVD	no	yes	yes	-20	B48,60,61	8	serological level	48	$7.67

Company	Product Name	PCR	FDA	DNA Ex	tag	alqt	°C	HLA Loci/allele group	primer sets	resolution	typings/ kit	cost/ typing
	Micro SSP HLA-A Locus Specific	yes	IVD	no	yes	yes	-20	HLA-A*01-80		serological level	16	$23.00
	Micro SSP HLA-B Locus Specific	yes	IVD	no	yes	yes	-20	HLA-B*07-82		serological level	8	$46.00
	Micro SSP HLA-C Locus Specific	yes	IVD	no	yes	yes	-20	HLA-Cw*01-18		serological level	16	$23.00
	Micro SSP DRB DQB Generic	yes	IVD	no	yes	yes	-20	DRB1,3,5 DQB1	32	serological level	30	$30.67
	Micro SSP HLA-DRB Generic	yes	IVD	no	yes	yes	-20	DRB1,3,4,5	24	serological level	40	$23.00
	Micro SSP HLA-DRB 1,3,4,5HiRes	yes	IVD	no	yes	yes	-20	DRB1,3,4,5	96	98% of combinations	10	$92.00
	Micro SSP ABDR DNA Typing Tray	yes	IVD	no	yes	yes	-20	A, B, DR	96	serological level	10	$92.00
	Micro SSP DRB1*01	yes	IVD	no	yes	yes	-20	DRB1*0101-*0105	8	5/5	24	$15.33
	Micro SSP DRB1*03	yes	IVD	no	yes	yes	-20	DRB1*0301-*0312	16	912/12	24	$15.33
	Micro SSP DRB1*04	yes	IVD	no	yes	yes	-20	DRB1*0401-*0432	32	32/32	12	$30.67
	Micro SSP DRB1*07,09,10	yes	IVD	no	yes	yes	-20	DRB1*07,*09,*10	8	5/5	24	$15.33
	Micro SSP DRB1*08+1415	yes	IVD	no	yes	yes	-20	DRB1*0801-*0815, *1415	24	22/22	16	$23.00
	Micro SSP DRB1*11	yes	IVD	no	yes	yes	-20	DRB1*1101-*1135	32	35/35	12	$30.67
	Micro SSP DRB1*12	yes	IVD	no	yes	yes	-20	DRB1*1201-*1205	11	5/5	24	$15.33
	Micro SSP DRB1*13	yes	IVD	no	yes	yes	-20	DRB1*1301-*1334	32	34/34	12	$30.67
One Lambda	Micro SSP DRB1*14	yes	IVD	no	yes	yes	-20	DRB1*1401-*1432	32	32/32	12	$30.67
	Micro SSP DRB1*05	yes	IVD	no	yes	yes	-20	DRB1*1501-*1508	12	8/8	24	$15.33
	Micro SSP DRB1*16	yes	IVD	no	yes	yes	-20	DRB1*1601-*1605, *1607,*1608	12	7/7	24	$15.33
	Micro SSP DRB3	yes	IVD	no	yes	yes	-20	DRB3*	16	16/16	24	$15.33
	Micro SSP DRB4	yes	IVD	no	yes	yes	-20	DRB4*	11	8/8	24	$15.33
	Micro SSP DRB5	yes	IVD	no	yes	yes	-20	DRB5*	16	12/12	24	$15.33
	Micro SSP DQB1	yes	IVD	no	yes	yes	-20	DQB1*0201-0402	32	30/30	12	$30.67
One Lambda	All products are also available w/o PCR license for 35% off list price											
Pel-Freez	ABC SSP UniTray	no	RUO	avail	no	yes	-20	HLA A,B,C	96		10	$60.00
	AB/DR/DQ SSP	no	RUO	avail	no	yes	-20	DR1-18,103 ,DR51,DR52,DR53	96		10	$70.00

Company	Product Name	PCR	FDA	DNA Ex	taq	alqt	°C	HLA Loci/allele group	primer sets	resolution	typings/ kit	cost/ typing
	AB/DR SSP UniTray	no	RUO	avail	no	yes	-20	DRB1,3,5	96		10	$70.00
	A Locus SSP UniTray	no	RUO	avail	no	yes	-20	HLA-A*01-*8001	24	30/87	30	$15.00
	B Locus SSP UniTray	no	RUO	avail	no	yes	-20	HLA-B*07-B*8201	96	72/119	20	$22.50
	C Locus SSP UniTray	no	RUO	avail	no	yes	-20	HLA-Cw*0102-Cw*1802, B*8201	24	22/41	30	$15.00
	DR/DQ SSP UniTray	no	RUO	avail	no	yes	-20	DR1-18,103 ,DR51,DR52,DR53 DQB1*02,05,06,07,08,09,04	40		20	$25.00
	DR SSP UniTray	no	RUO	avail	no	yes	-20	DR1-18,103 ,DR51,DR52,DR53	24		40	$15.00

SSOP

Company	Product Name	PCR	FDA	DNA Ex	taq	°C	Format	HLA Loci/allele group	primer sets/ probes	resolution	typings/kit	cost/ typing
Abbott	LiPA A	yes	ASR	no	yes	4	Rev Line Blot	HLA-A*01-*8001		58/92	20	$44.00
	LiPA B	yes	ASR	no	yes	4	Rev Line Blot	HLA B		175/213	20	$44.00
	LiPA DRB 1	yes	ASR	no	yes	4	Rev Line Blot	DRB1,3,4,5	2/34	113/136	20	$44.00
	LiPA DRB decoder	yes	ASR	no	yes	4	Rev Line Blot	DRB1,3,4,5				
	LiPA DRB1*04	yes	ASR	no	yes	4	Rev Line Blot	DRB*0401-*0421	2/21	20/21		
	LiPA DPB	yes	ASR	no	yes	4	Rev Line Blot	DPB	2/26	58/61	20	$44.00
	LiPA DQB	yes	ASR	no	yes	4	Rev Line Blot	DQB	2/23	25/26	20	$44.00
Biotest	DRB ELPHA	yes	IVD	no	yes	4	ELISA	DRB1,3,4,5	2/24	69/136	12	$47.00
	HiRes DRB ELPHA	yes	RUO	no	yes	4	ELISA	DRB1,3,,4,5	4/64		6	$94.00
	DQB ELPHA	yes	IVD	no	yes	4	ELISA	DQB1*02-*0402	2/22	24/25	12	$47.00
	ELPHA DR3/11/6	yes	RUO	no	yes	4	ELISA	DRB1*03,11,06	2/56		12	$46.00
	ELPHA DR4	yes	RUO	no	yes	4	ELISA	DRB1*04	2/32		12	$36.00
	ELPHA DR2	yes	RUO	no	yes	4	ELISA	DRB1*15,16	3/24		12	$36.00
	ELPHA DR 8+12	yes	RUO	no	yes	4	ELISA	DRB1*08,12,1404	2/32		12	$36.00
	ELPHA DRB3	yes	RUO	no	yes	4	ELISA	DRB3*	2/24		12	$29.00
Dynal	RELI DRB	yes	RUO	no	yes	4	Rev Line Blot colormetric	DRB1,3,4,5	2/29	79/156	50	$28.02
	RELI DQB	yes	RUO	no	yes	4	Rev Line Blot colormetric	DQB1*	2/25	22/25	50	$28.68
	RELI A Locus	yes	RUO	no	yes	4	Rev Line Blot colormetric	HLA A	2/35	66/86	50	$28.02
	RELI B Locus	yes	RUO	no	yes	4	Rev Line Blot colormetric	HLA B	4/56	192/237	50	$37.34

							SSOP					
Company	Product Name	PCR	FDA	DNA Ex	tag	°C	Format	HLA Loci/allele group	primer sets/ probes	resolution	typings/kit	$14.25
Gen Trak	CQuentials	no	IVD	no	no	4/-20	ELISA	DRB1,3,4,5	4/32	51/118	12	$41.20
	CQuentials DQ	no	IVD	no	no	4/-20	ELISA	DQB1*0501-0402	2/16	22/22	24	$20.62
Lifecodes	HLA-A high resolution	yes	ASR	avail	yes		Dot Blot chemiluminescent or colormetric	HLA-A high resolution	2/52		100	$20.75
	HLA-B low resolution	yes	ASR	avail	yes		Dot Blot chemiluminescent or colormetric	HLA-B low resolution	32/38		100	$16.75
	HLA-B high resolution	yes	ASR	avail	yes		Dot Blot chemiluminescent or colormetric	HLA-B high resolution	2/60		100	$22.25
	HLA-A NMDP SET	yes	ASR	avail	yes		Dot Blot chemiluminescent or colormetric	HLA-A NMDP SET	2/30		100	$15.00
	HLA-B NMDP SET	yes	ASR	avail	yes		Dot Blot chemiluminescent or colormetric	HLA-B NMDP SET	2/46		100	$19.00
	HLA-C low resolution	yes	ASR	avail	yes		Dot Blot chemiluminescent or colormetric	HLA-C low resolution	2/22		100	$12.50
	HLA-C high resolution	yes	ASR	avail	yes		Dot Blot chemiluminescent or colormetric	HLA-C high resolution	2/38		100	$16.75
	DRB NMDP set	yes	ASR	avail	yes		Dot Blot chemiluminescent or colormetric	DRB NMDP set	2/31		100	$15.25
	DRB low resolution	yes	ASR	avail	yes		Dot Blot chemiluminescent or colormetric	DRB low resolution	2/22		100	$12.50

							SSOP					
Company	Product Name	PCR	FDA	DNA Ex	tag	°C	Format	HLA Loci/allele group	primer sets/ probes	resolution	typings/kit	$14.25
Lfecodes	DRB medium resolution	yes	ASR	avail	yes		Dot Blot chemiluminescent or colormetric	DRB medium resolution	2/37		100	$16.50
	DRB high resolution	yes	ASR	avail	yes		Dot Blot chemiluminescent or colormetric	DRB high resolution	2/53		100	$20.25
	DR 1 group	yes	ASR	avail	yes		Dot Blot chemiluminescent or colormetric	DR 1 group	2/6		100	$6.50
	DR 2 group	yes	ASR	avail	yes		Dot Blot chemiluminescent or colormetric	DR 2 group	2/17		100	$10.75
	DR 4 group	yes	ASR	avail	yes		Dot Blot chemiluminescent or colormetric	DR 4 group	2/27		100	$14.25
	DR 52 group	yes	ASR	avail	yes		Dot Blot chemiluminescent or colormetric	DR 52 group	2/42		100	$17.50
	DQB	yes	ASR	avail	yes		Dot Blot chemiluminescent or colormetric	DQB	2/21		100	$12.25
	DPB	yes	ASR	avail	yes		Dot Blot chemiluminescent or colormetric	DPB	2/28		100	$14.25

Procedure Notes

PCR Royalty License

Yes – use of these products is covered by limited non-transferable license from F. Hoffman-LaRoche or Roche Molecular Systems Inc. for HLA Typing
No – use of these products do not convey any license to use the PCR process.

FDA

RUO – RUO Only
IVD – In-Vitro diagnostic use approved
ASR- Analyte specific reagent

DNA Ex

yes – DNA extraction kit is included in price of the test
no – DNA extraction kit is not included in the price of the test, nor does company offer a DNA extraction product
avail – DNA extraction kit is not included in he price of the kit, but the company does offer a DNA extraction kit for an extra cost

taq

yes – taq polymerase is included in the price of the test
no- taq polymerase is not included in the price of the test

alqt

yes – a master mix less DNA template or taq polymerase is pre-aliquoted into microtube strips or plates

°C

storage temperature of products. Some products store master mixes and PCR microtubes at two different temperatures

cost/typing

list price (January 1, 1999), most vendors offer volume discounts

References

1. Abbott Laboratories 100 Abbott Park Rd., Abbott Park, IL 60064 1-800-323-9100 www.abbott.com
2. Biotest Diagnostics Corp. 66 Ford Rd., Suite 131 Denville NJ 07834 1-800-522-0090 www.biotest.com
3. Biosynthesis Inc. 612 E. Main St Lewisville, Tx 75057 1-800-227-0627 www.biosyn.com
4. Dynal, Inc 5 Delaware Drive, Lake Success, NY 11042 1-800-638-9416 www.dynal.no
5. GenoVision 140 Arrandale Blvd., Exton, PA 19341 1-888-559-0888 fax 1-610-280-9532 www.genovision.com
6. Gen Trak, Inc. 5100 Campus Drive, Plymouth Meeting, PA 19462 1-800-221-7407
7. Lifecodes Corporation, 550 West Ave Stamford CT 06902 1-800-543-3263 www.lifecodes.com
8. One Lambda Inc. 21001 Kittridge, St Canoga, CA 91303 1-800-822-8824 www.onelambda.com
9. Pel Freez Clinical Systems 9099 North Deerbrook Trail, Brown Deer, WI 53223 1-800-558-4511 www.pel-freez.com

Analysis of HLA Class I Alleles via Direct Sequencing of PCR Products

Jin Wu, Sue Bassinger, Barbara B. Griffith, and Thomas M. Williams

Purpose

DNA sequencing is a powerful and general method for identifying HLA Class I alleles. Laboratories may employ this technique for high resolution allele identification for diagnostic and research applications or to resolve typing problems encountered with low resolution serologic or DNA-based methods.

DNA sequencing relies upon the use of a polymerase to create a nested series of DNA fragments which are complementary to a DNA template. A DNA polymerase can copy a single stranded template in a 5' to 3' direction beginning at a hybridized sequencing primer if it is supplied with the four deoxynucleotides (dNTPs) and appropriate cofactors. For a DNA template *n* nucleotides in length, a population of DNA fragments of size *n-1, n-2, n-3, n-4, n-5* and so on whose 3' ends terminate at every nucleotide position complementary to the DNA template can be synthesized if the dNTPs in the reaction are mixed with small amounts of dideoxynucleotides (ddNTPs). Chance incorporation of ddNTPs into a particular growing DNA strand makes impossible the addition of another nucleotide to that strand. Each DNA fragment's 3'-end will be composed of either an adenine, guanine, thymine, or cytosine nucleotide. Fragments terminating in each of the four bases are labeled with one of four distinct fluorescent dyes linked to either the ddNTPs or to the sequencing primer employed. Since the fragments differ in size from each other in one nucleotide increments, they can be resolved via high resolution gel electrophoresis and the fluorescence of each fragment interrogated by a laser. In this way, the original sequence of the DNA template can be deduced using a data management system with appropriate sequencing software.

The most convenient DNA template for this procedure is a polymerase chain reaction product prepared from a patient's or donor's genomic DNA. Ideally, the PCR product should include all regions of the Class I gene known to differ in sequence from allele to allele. At least exons 2 and 3 should be represented since knowledge of the sequence of these exons is usually required to make unambiguous allele calls. Direct DNA sequencing allows all known alleles at a particular locus to be precisely identified and new alleles to be recognized with a general strategy as long as the relevant polymorphic nucleotides lie within the PCR products prepared. In contrast, other HLA typing approaches require large numbers of hybridization probes or selective PCR primers, which sometimes must be modified when new alleles are described. The combined availability of instruments for fluorescent detection of nested DNA sequencing ladders, advanced software for allele recognition, and chemistries allowing semi-automated thermal cycle sequencing of crude double stranded products PCR enables direct sequencing to be used as a means for routine identification of Class I alleles.

Specimen

Any source of nucleated cells from which genomic DNA may be extracted and isolated is appropriate for Class I allele identification by sequencing. In practice, most laboratories and clinics will find that 3-5 ml of ACD-A or EDTA anticoagulated peripheral blood is convenient and adequate for DNA sequencing studies. However, other sample sources such as frozen tissue, buccal mucosa swabs, and paraffin embedded tissue may also be employed. DNA in paraffin blocks is usually sufficiently degraded and crosslinked enough so as to make generation of PCR products encompassing more than one of the Class I exons difficult.

Samples must be collected in clean, sterile containers clearly labeled with the patient name, identifying number, and date and transported to the laboratory at room temperature. If transit times are long (4-48 hours), and ambient temperatures are very high or low, transportation in an insulated container on wet ice is recommended.

Samples are unacceptable if they are improperly labeled.

Reagents and Supplies

Reagents are stored at room temperature unless specified.

Polymerase Chain Reaction

10X PCR buffer for HLA-A and -C: 150 mM ammonium sulfate, 0.5 M Tris-HCl (pH 8.8), 0.5 mM EDTA (pH 8.0), 15 mM $MgCl_2$, 0.1% gelatin, 100 mM β-mercaptoethanol. Store as aliquots at -20° C.

10X PCR Buffers for HLA-B:

Buffer A: 500 mM KCl, 100 mM Tris HCl pH 8.3, 12.5 mM $MgCl_2$.

Buffer B: 104 mM Tris-HCl, pH 8.5; 15mM $MgCl_2$; 250mM KCl; 50nM EDTA.

Buffer C: 100mM Tris-HCl, pH 9.2; 500 mM KCl; 25mM $MgCl_2$

Store as aliquots at – 20° C

Dimethyl sulfoxide

PCR Primers: 250mM stock solutions in H_2O at -70° C

dNTPs: Stock solutions at 10mM stored at -20° C

Taq polymerase(5U/ µl, Perkin Elmer) Store at -20° C

Sequencing Reactions

HLA-A:

Dye Primer Core Reagents containing dNTP/ddNTPs, TaqFS polymerase, and buffer. Store at -20° C (Perkin Elmer)

Sequencing Primer stock. Store at -20° C

HLA-B and HLA-C:

Big Dye™ Primer Cycle Sequencing Ready Reaction -21 M13 containing labeled M13 sequencing primer, dNTP/ddNTPs, TaqFS polymerase, and buffer. Store at -20° C. (Perkin Elmer)

Big Dye™ Primer Cycle Sequencing Ready Reaction M13 Rev containing labeled M13 sequencing primer, dNTP/ddNTPs, Taq FS polymerase, and buffer. Store at -20° C (Perkin Elmer)

Sequencing Gel

Deionized Formamide 5X loading buffer: Prepare 5:1v/v of formamide to Blue dextran (mg/ml)/EDTA (25 mM, pH 8.0, Perkin Elmer) solution. Store formamide in dark at 4° C and Blue Dextran/EDTA at 4° C.

Urea, molecular biology grade, (Kodak)

TEMED (Amresco)

10% Ammonium persulfate (w/v) in dH_20 (make fresh weekly, store at 4° C)

Long Ranger™ acrylamide gel solution, 50% stock (FMC)

0.22 µM nylon filters for filtering sequencing acrylamide mix (Corning)

Mixed bed, ion exchange resin IRN-150 (Alfa Aesar) for deionizing gels

Microconcentrated cleaning solution for cleaning sequencing gel glass plates (International Products Corporation)

10X Tris/borate/EDTA electrophoresis buffer (TBE): 108.0 g Tris base, 55.0 g Boric Acid, 8.3 g Na_2EDTA and q.s. with H_2O to 1 liter, pH 8.3.

Instrumentation/Equipment

Spectrophometer

Low speed centrifuge

Microcentrifuge

Ultraviolet light bathed hood for PCR set-up

Pre- and post-PCR equipment and facilities

Micropipettors (volumes 1-250 µl)

Refrigeration/freezer capacity at 4°, -20°, and -70° C

Thermalcycler

Agarose horizontal gel electrophoresis equipment and power supply

Agarose gel electrophoresis documentation equipment including a UV transilluminator

Speed-Vac or equivalent centrifuge

Heat blocks

Automated DNA sequencer (this procedure is compatible with an ABI-Perkin Elmer 377 instrument) with glass plates and gel casting equipment

HLA-A, -B, and -C allele identification software with an appropriate computer

Calibration

The numerous instruments necessary for this procedure must be calibrated by trained personnel on a regular basis. The quality control program of the laboratory should provide for comprehensive calibration and assessment of the pipettors, refrigerators, thermalcyclers, centrifuges, and other equipment listed above (See Section VII, this manual).

Quality Control

This is a multi-step procedure with many opportunities for problems. A laboratory performing direct sequencing as part of its histocompatibility services should have a comprehensive quality control and assurance plan (Section VII).

1. There must be a comprehensive protocol for control and monitoring of contamination of the pre-PCR area with genomic DNA and PCR products.
2. PCR products prepared as templates for sequencing reactions should be free of non-specific amplification products as assessed by gel electrophoresis and must be locus specific to generate high quality electropherograms.
3. Care must be taken to ensure that reagents are pure and water is of high quality for the PCR, sequencing, and gel electrophoresis steps described.
4. If electropherograms produced have high background and a low signal to noise ratio, the same PCR product can be resequenced. If resequencing of the original PCR product fails again to generate an interpretable electropherogram, then new PCR product should be synthesized and sequenced.
5. Laboratory personnel should be capable of manually reading electropherograms and assigning alleles to edit sequences, to validate the performance of software used and to assess lower quality data from which available software cannot successfully assign alleles.

Procedure

A. Isolation of Genomic DNA

Methods for extraction and isolation of genomic DNA from patient samples are described elsewhere in this manual (Section V.A.1). The concentration and purity of isolated DNA should be evaluated with a spectrophotometer before proceeding to the PCR (See Section V.D.1, this manual).

B. Preparation of HLA-A, -B, and -C PCR Products as Sequencing Templates

This protocol describes locally modified methods for obtaining HLA-A, -B, and -C sequences that allow for allele identification. Several commercial kits for HLA sequencing are now available which may be an attractive alternative to laboratories.

The HLA-A gene exons 1-3 and introns 1-2 are amplified with locus specific intronic primers and sequenced using Dye Primer Taq FS Core Kit (PE) cycle sequencing reagents with fluorescent labeled primers that anneal internally within the PCR template.

A strategy of both group-specific and generic amplification is used for the B locus. A generic B locus amplification product and five independent HLA-B group specific amplifications are used to obtain HLA-B exon 2 and 3 sequences. Serologic, SSP-PCR or sequence data from the generic amplification reaction may be used to guide the choice of primers for group specific amplification. Generic amplification of locus B with primers M20EX2 and M18CIN2.166G allows preparation of templates for the determination of exon 2 sequence. The five group specific reactions described in the tables below allow preparation of sequencing templates from the subgroups of HLA-B alleles. These templates allow determination of exon 3 sequence. HLA-B PCR primers are synthesized with 5' tails to provide -21M13 and M13 reverse sequencing primer annealing sites. PCR products are sequenced with Dye Primer Taq FS(PE) cycle sequencing reagents.

The HLA-C gene exons 2-3 and intron 2 are amplified with locus specific intronic primers with 5' tails which provide annealing sites for fluorescent labeled -21M13 and M13 reverse sequencing primers. The PCR products are sequenced with Dye Primer Taq FS(PE) cycle sequencing reagents.

HLA-A and -C locus Specific Amplification Reactions

Note: This procedure is written for a Perkin Elmer 9600 instrument – results obtained with other models and vendors must be validated prior to actual patient testing

1. To set up polymerase chain reactions, work in a static hood which has been bathed in UV light. Remember to turn off the UV light before working in the hood. Always wipe working surface with 10% bleach following by 70% ETOH before and after PCR set up. Label autoclaved 0.2 ml thermalcycler tubes.
2. Thaw PCR reagents on ice (except Taq polymerase) and use immediately.
3. In the static hood prepare a volume of master amplification mix sufficient for the number of DNA samples to be amplified by adding, in order, sterile D-H_2O, 10X buffer, DMSO, dNTPs, and the PCR primers. Vortex thoroughly, add Taq polymerase, and mix gently with the pipettor. The mastermix should be prepared so that the final concentrations of the reagents in 100 µl of PCR sample are 1X buffer, 5%v/v DMSO, 0.2mM dNTPs, 0.2 µM primers, and 2.5 U/100 µl Taq polymerase.
4. Aliquot 98 ml of master mix into each reaction tube. Add 2 µl of each genomic DNA sample suspended at 0.25 µg/ µl to each tube. For the negative (no DNA) control, add 2 µl autoclaved dH_2O to a tube.

5. Place the PCR reactions in the thermalcycler programmed as follows:
   96° C for 5 min
   94° C for 22 sec, 65° C for 50 sec, 72° C for 30sec (x 30 cycles)
   94° C for 22 sec, 65° C for 50 sec, 72° C for 10 min (1 cycle)
   Hold at 4° C

HLA-B Locus Generic and Group Specific Amplification Reactions

1. To set up polymerase chain reactions, work in a static hood which has been bathed in UV light. Remember to turn off the UV light before working in the hood. Always wipe working surface with 10% bleach following by 70% ETOH before and after set up PCR. Label autoclaved 0.2 ml thermalcycler tubes.
2. Thaw PCR reagents on ice (except Taq polymerase) and use immediately.
3. In the static hood prepare a volume of master amplification mix sufficient for the number of DNA samples to be amplified by adding, in order, sterile distilled H_2O, 10X buffer, dNTPs, and the PCR primers. Vortex thoroughly, add Taq polymerase, and mix gently with the pipettor. The mastermix should be prepared so that 100 µl amplification reactions contain 10 µl of 10X PCR buffer A, B, or C and 1 µM of the primers as specified in the table, 0.25 mM dNTPs, 2.5 U Taq polymerase, and 0.4-1.0 mg of genomic DNA. Optimal genomic DNA concentrations are 0.5 µg/100 µl for primers M20EX2/M18CIn2.166G, 1 µg/100 µl for primers MEE/M18CIN3, MMA/M18CIN3, MTK/M18CIN3, and MTE/M18CIN3, and 0.4 µg/100 µl for primers B#4B/B#C3.

   Note: For reactions with primers MB#4B/MB#C3 the PCR is optimal when the dNTP final concentration is 0.2 mM and primer concentrations are 0.68 µM.

   The MEE/M18CIN3 primers should be used with caution because preferential amplification has been observed.
4. Place the PCR reactions in the thermalcycler programmed as follows:
   96° C for 5 min
   96° C for 30 sec, 60°, 65°, or 68° C for 30 sec, 72° C for 60 sec (x 30 or 35 cycles)
   72° C for 10 min
   Hold at 4° C

   Note: Specific annealing temperatures and cycle numbers can be determined from the Table 1 for each primer pair.

Table 1: HLA-B Group Specific and Generic PCR Conditions

5' Primer	3' Primer	Product Size	Specificity	10X PCR Buffer	Annealing Temp. (°C)	Cycles
MEE	M18CIN3	680 bp	B*07, *08, *14, *1503, *1509, *1510, *1518, *1523, *1529, *1537, *27, *38, *39, *42, *48, *55, *56, *59, *67, *73, *81, *82	A	68	35
MTK	M18CIN3	680	B*40, *41, *44, *45, *47, *49, *50	A	65	35
MTE	M18CIN3	670	B*1522, *18, *35, *37, *51, *52, *53, *58, *78	A	60	30
MMA	M18CIN3	670	B*13, *1501-1502, *1504-1508, *1511-1517, *1519-1521, *1524-1525, *1526N, *1527-1528, *1530-1535, *1538, *46, *57	A	65	35
MB#4B	MB#C3	489	B*54, *55, *56, *59	B	65	35
M20Ex2	M18CIN2.166G	456	Generic Locus B	C	60	30

Note: Since the B*15 alleles are split among several group specific primers, sequences of newly described B*15 alleles should be inspected to determine which group specific primer will result in amplification.

Confirmation PCR Results

Assess the success and specificity of the PCR via electrophoresis of 10 ml of PCR products and 2 ml of 5X loading buffer on a 1% SeaKem agarose gel complete with ethidium bromide at 150 volts for 30 minutes. Use appropriate size markers and document the gel image. The HLA-A and -C products should have sizes of 1184 bp and 909 bp, respectively. HLA-B products have a variety of sizes depending on primer pairs employed (Table 1).

Table 2. HLA-A and -C PCR Primers

Locus	Primer	Sequence (5'-3')	Position (bp)
A	5A.2	CCCAGACGCCGAGGATGGCCG	5' flanking region 509-529
	3A.2	GCAGGGCGGAACCTCAGAGTCACTCTCT	Intron 3 1666-1693
C	M5CIn 1-61*	AGCGAGGG/TGCCCGCCCGGCGA	Intron 1 278-299
	M3CIn 3-12*	GGAGATGGGGAAGGCTCCCCACT	Intron 3 1166-1187

* Primers are tailed with annealing sites for either -21M13 or M13 reverse .

Table 3. HLA-B PCR Primers

Primer*		Sequence (5'-3')	Position (bp)
5'	MEE	CGCCGCGAGTCCGAGAGA	Exon 2 116-133
5'	MTK	CGCCACGAGTCCGAGGAA	Exon 2 116-133
5'	MTE	CCGAGGACGGAGCCCCGG	Exon 2 125-143
5'	MMA	CCGAGGATGGCGCCCCGG	Exon 2 125-143
5'	MB#4b	CGCCGCGAGTCCGAGAG	Exon 2 116-133
5'	M20EX2	GCTCCCACTCCATGAGGTAT	Exon 2 1-20
3'	M18CIN3	CCCACTGCCCCTGGTACC	Exon 3 276-Intron 317
3'	MB#C3	ATCCTTGCCGTCGTAGGCT	Exon 3 77-95
3'	M18CIN2.166G	AAATGAAACCGGGTAAAC	Intron 2 166-186

* All the 5' primers are tailed with -21M113 primer, all the 3' primers are tailed with M13 reverse primer.

C-1. Direct DNA Sequencing of HLA-A PCR Products

1. Label an appropriate number of microcentrifuge tubes and prepare worksheets.
2. Dilute the HLA-A PCR product from the reactions above to be sequenced with dH_2O (1:4v/v). Smaller dilutions can be made if the PCR band seen on gel electrophoresis was relatively weak.
3. Prepare appropriate number of PCR tubes and a tray for the thermalcycler allowing 4 tubes per sample to be sequenced.
4. Prepare A, C, G, and T base specific sequencing master mixes on ice:

Table 4. Preparation of Master Mixes for HLA-A Locus

Reagent	A Reaction	C Reaction	G Reaction	T Reaction
5X buffer	1 µl	1 µl	2 µl	2 µl
DNTP/ddNTP mix	1 µl	1 µl	2 µl	2 µl
Sequencing Primer (0.2pmol)	1 µl	1 µl	2 µl	2 µl
TaqFS polymerase	1 µl	1 µl	2 µl	2 µl
Total volume	4 µl	4 µl	8 µl	8 µl

Note: The TaqFS enzyme used in the master mixes is prepared by diluting 1 µl of TaqFS in 1 µl of 5X buffer and 5 µl H_2O.

5. Place 4 µl A mix and C mix and 8 µl G mix and T mix, respectively, in 4 PCR tubes for each sample to be sequenced. Dispense 1 µl diluted DNA template to A and C PCR tubes and 2 µl diluted DNA template to G and T PCR tubes.
6. Cap tubes and place in thermalcycler programmed to run the following profile:
   98° C for 9 min for 1 cycle
   96° C for 5 min followed by 68° C for 1 min for 30 cycles
   Hold at 4° C

Table 5. Sequencing Primers for HLA-A

Primer	Sequence (5'-3')	Position
B3.6 (Reverse)	CACTCACCGGCCTCGCTCTGG	Exon 2 329-343 to Intron 2 1-7
A5.10 (Forward)	GGGCTCGGGGGACT/CGGG	Intron 2 35-52

C-2. Direct DNA Sequencing of HLA-B PCR Products

1. Label an appropriate number of microcentrifuge tubes and prepare worksheets.
2. Dilute the HLA-B PCR product from the reactions above to be sequenced with dH_2O (1:5v/v). Smaller dilutions can be made if the PCR band seen on gel electrophoresis was relatively weak. Crude, double stranded PCR products can be sequenced with this method. However, it is recommended to purify the PCR template with the ancillary procedure below.
3. Prepare appropriate number of PCR tubes and a tray for the thermalcycler allowing 4 tubes per sample to be sequenced.
4. Prepare A, C, G, and T base specific sequencing master mixes on ice and dispense 1 µl diluted DNA template to each tube.

Table 6. Preparation of Master Mixes for HLA-B Locus

Reagent	A Reaction	C Reaction	G Reaction	T Reaction
Sequencing mix	4 µl	4 µl	4 µl	4 µl
Diluted PCR product	1 µl	1 µl	1 µl	1 µl
Total volume	5 µl	5 µl	5 µl	5 µl

5. Cap tubes and place in thermalcycler programmed to run the following profile:
   96° C for 10 sec
   55° C for 5 sec followed by 72° C for 1 min for 15 cycles
   96° C for 10 sec followed by 72° C for 1 min for 15 cycles
   Hold at 4° C

Ancillary Procedure: PCR Products Purification Prior to Sequencing Reactions

Most Class I PCR products can be diluted and used as crude templates for DNA sequencing as described in Step #2 above. Some products, especially HLA-B amplicons, give higher quality electropherograms if they are purified prior to the sequencing reactions.

1. PCR products are purified using QIAquick™ PCR Purification kit (Qiagen) including buffers PB and PE.
2. Add 5 volumes of buffer PB to 1 volume of the PCR reaction and mix, for example 250 µl buffer PB to 50 µl PCR reaction.
3. Place a QIAquick spin column in a 2 ml collection tube and apply the sample.
4. Centrifuge according to the manufacturer's instructions for 1 min.
5. Discard flow-through and place QIAquick column back into the same tube.
6. To wash, add 0.75 ml buffer PE to QIAquick column and centrifuge 1 min.
7. Discard flow-through and place QIAquick column back in the same tube. Centrifuge QIAquick column for additional 1 min at maximum speed.
8. Place QIAquick column in a 1.5 ml microfuge tube.
9. To elute DNA, add 50 µl autoclaved dH_2O pH 7.5-8.0 to center of the QIAquick column and centrifuge for 1 min. To increase DNA concentration, add 30 µl elution buffer, let stand for 1 min, and then centrifuge.
10. Purified PCR products can be dried in a Speed-Vac and rediluted in H_2O if it is necessary to concentrate them for the sequencing reactions.

C-3. Direct DNA sequencing of HLA-C PCR products

1. Label an appropriate number of microcentrifuge tubes and prepare worksheets.
2. Dilute the HLA-C PCR product from the reactions above to be sequenced with dH_2O (1:3v/v). Smaller dilutions can be made if the PCR band seen on gel electrophoresis was relatively weak.
3. Prepare appropriate number of PCR tubes and a tray for the thermalcycler allowing 4 tubes per sample to be sequenced.
4. Prepare A, C, G, and T base specific sequencing master mixes on ice and dispense 1 µl diluted DNA template to each tube.

Table 7. Preparation of Master Mixes for HLA- C Locus

Reagent	A Reaction	C Reaction	G Reaction	T Reaction
Sequencing mix	4 µl	4 µl	4 µl	4 µl
Diluted PCR product	1 µl	1 µl	1 µl	1 µl
Total volume	5 µl	5 µl	5 µl	5 µl

5. Cap tubes and place in thermalcycler programmed as follows:
   96° C for 1 sec followed by 55° C for 10 sec and 72° C for 1 min for 15 cycles
   96° C for 10 sec followed by 72° C for 1 min for 15 cycles
   Hold at 4° C

D. Precipitation and Resuspension of DNA from Sequencing Reactions

1. Remove sequencing reactions from thermalcycler and combine the four reactions into a 1.5ml microcentrifuge tube containing 80 µl 95% ethanol to precipitate DNA.
2. Spin at 14000 rpm in microcentrifuge at 4º C for 20 minutes.
3. Aspirate supernatant from DNA pellets and dry in Speed-Vac until no ethanol remains.
4. Prepare a 5:1v/v mixture of formamide/loading buffer, adding 6 µl to the DNA pellets in each tube.
5. Denature DNA by placing tubes in a 95° C heating block for 2 minutes and immediately place on ice. Load the reactions on the automated sequencer within 30 min or store at -20° C.

E. Electrophoresis and Detection of Sequencing Reaction Products

Note: This protocol has been optimized for use with a PE-ABI Prism™ 377 DNA Sequencer. Other automated fluorescent sequencing systems and manual systems employing radioactive labels are available for this purpose and this procedure would have to be validated for those instruments.

Automated DNA sequencing systems are capable of size fractionation and detection of DNA sequencing reaction products in order to determine the nucleotide sequence of a DNA fragment of interest. An automated sequencing system consists of an electrophoresis instrument linked to a computer capable of running the necessary software for data collection and data analysis. Sequencing analysis software can analyze the raw data produced by the instrument.

Dye-labeled DNA fragments are electrophoresed through an acrylamide gel and are separated according to size. Near the bottom of the gel they pass through a region where a laser beam continuously scans the gel. The laser excites the fluorescent dyes linked to the DNA fragments, and they emit light at wavelengths with specific maxima for each dye. The light is collected and separated according to wavelength by a spectrograph. The data collection software collects the light intensities using software-selectable filters and stores them as electrical signals for eventual processing by the instrument's software to identify sequences. Additional software allows comparison of sequences of the electrophoresed amplicons with known Class I sequences for specific identification of alleles.

Acrylamide Gel Preparation

The quality of sequencing data is highly dependent on the use of high quality reagents and distilled deionized water for gel preparation. All solutions must be filtered in order to remove particulate matter that may fluoresce or scatter light. Moreover, clean lab gloves must be worn to avoid transferring fluorescent contaminants from the investigator's hands to the glass plates.

Denaturing Gel

1. Dissolve 18 g urea in 26 ml glass distilled, deionized water and mix with 5 ml FMC Long Ranger™ gel solution (50% stock solution).
2. Add 1 g Amberlite (IRN-150), stirring for 5 minutes to deionize the gel mix.
3. Filter the acrylamide/urea mix using a 0.22µm filter. Attach a vacuum source to pull liquid through the filter. Degas the mix for 5 minutes.
4. Add 5 ml of 10x TBE (108.0g Tris base, 55.0g Boric Acid, 8.3g $Na_2EDTA\ 2H_2O$) to the gel mix. (The gel mix can be held up to 12 hours before use at this point in the procedure).
5. To polymerize the gel, add 250 µl of freshly prepared 10% ammonium persulfate and 22.5 µl TME buffer. Swirl gently to mix and initiate polymerization.
6. With the clean glass plates placed on the gel loading apparatus and the 0.22mm gel spacers properly placed on the bottom plate, pour the gel slowly while sliding the top plate over the bottom plate. Avoid trapping any air bubbles between the two plates as they will distort the sample path, which affects lane tracking.

   Note: With each use, orient the same side of each glass plate to the inside. After the first use, the front plate has a hydrophobic area where the buffer chamber gasket made contact with the plate. To avoid problems pouring the gel, use the top plate in the same configuration each time.
7. Align the plates at the bottom, insert the casting comb, and attach 2 clamps over the casting comb at the top of the plates. Clamp the edges of the plates with 3 clamps on each side of the plates. Proper polymerization of the gel should occur within two hours. Gels should be run within 24 hours of pouring, since a gel older than one day will begin to show signs of degradation.

8. After the gel has polymerized, remove the casting comb and the clamps from the gel and wash the exterior surfaces of the plates. The glass must be clean where the laser reads the gel.
9. Place the plates into the gel cassette and clamp in place. Slide the shark's tooth comb between the top of the plates, until the tips of the teeth just touch or slightly depress the surface of the gel. Do not attempt to withdraw the comb or the samples will leak into adjacent wells.
10. Place the lower buffer chamber into the bottom shelf of the sequencer.
11. Load the cassette with the gel into the sequencer, clamp into place, and close the door.
12. Restart the computer.

Preparing the Run

13. The data collection should automatically open when the computer is restarted. Choose **File, New, Sequence Run**. This will open a new run window.
14. Click **Plate Check** after selecting the plate check module. The laser scans the plates without electrophoresis to detect any unwanted fluorescent material in the read region.
15. Watch the scan window that appears on the screen. The scan window should show a relatively flat line across the screen in each of the four colors. If the scan lines are flat, the plates are clean. If there are peaks in the scan window, cancel the run, clean the plates, and run another plate check. If the peaks do not disappear after cleaning the plates, the gel mixture or buffer may contain air bubbles or contaminating particles. In order to use the gel, avoid loading samples in the lanes where the peaks appear. Use the ABI table in the appendix of this protocol to determine which lanes are contaminated and do not load samples in those lanes.
16. Fill the upper buffer chamber with approximately 600 ml 1X TBE buffer. Then fill the lower buffer chamber to its capacity. Flush out the wells with buffer using a plastic pipette or syringe. Install the lid on the upper buffer chamber.
17. Attach the front heat-transfer plate under the upper buffer reservoir, securing it with the plate clamps. Attach the quick-connect water lines and the ground cable. Plug in the electrode cables.
18. Pre-run the gel to equilibrate the temperature.(Click the **PreRun** button) The gel should be at 51° C before loading samples to ensure adequate DNA denaturation.(Select **Status** from the Window menu in order to monitor temperature). Prerunning also removes mobile ions from the gel and prevents the power surge that could ensue if high voltage was suddenly applied. No windows appear during a pre-run because the instrument performs electrophoresis without starting data collection.
19. Create a sample sheet. Select **New** from the File menu. Click on **Sequence Sample**. Enter the necessary information, including sample name, mobility file and matrix. When finished, select **Save As** from the File menu and enter SS (Sample Sheet) with the date the gel is being run, e.g., SS 980514.

Electrophoresis

20. Resuspend DNA samples in 6 µl of loading buffer (5:1 deionized formamide: 25mM EDTA with 50 mg/ml blue dextran) and vortex. Heat samples at 95° C for two minutes, holding on ice until ready to load.
21. Click **Pause** in the Run window. (Pause will stop electrophoresis but maintain the temperature of the gel while loading). Flush all wells with the 1X buffer.
22. Load formamide in the well to the left of the first sample lane, and in the well to the right of the last sample lane. Formamide in the buffer helps focus the bands in the first and last lanes.
23. Load 1.0-2.0 µl of each sample into each of the odd-numbered wells. When running many samples, it is important to load samples in alternate wells. Electrophorese briefly, rinse all wells, and then load the remaining samples onto the gel. Since the automatic lane tracker in the analysis software needs to have discrete spaces between samples to identify the lanes properly, load samples in alternate lanes as follows:
    a. Click **Resume** in the Run window after loading first set
    b. Electrophorese for two minutes to allow the samples to enter the gel.
    c. Click **Pause** in the Run window.
    d. Flush all wells with 1X buffer to remove any residual formamide from previously loaded wells.
    e. Load the even-numbered wells and electrophorese for two minutes.
    f. Click **Cancel** in the Run window and flush all wells with 1X buffer.
    g. Click **Run** to begin the run.
24. When the dialog box appears, name the gel with the current date; e.g., Gel 980514 and click OK.
25. After the gel has finished running, turn off the instrument, then disconnect the electrode leads, the front heat-transfer plate ground cable and water lines.
26. Remove the front heat-transfer plate, then carefully remove the cassette holding the gel plates. Lift up as pull the cassette is pulled out or the bottom buffer chamber will be pulled out at the same time.
27. Remove the upper buffer chamber from the cassette and the lower buffer chamber from the instrument. Rinse with deionized water. Also rinse the cassette with deionized water to remove any salt buildup.
28. Gently push a thin blade almost all the way in at the bottom of the plates (**Do not** use the top notches as they are likely to break off.) and pry the plates apart. Remove the comb and gel spacers. Lay two paper towels or a large Kimwipe™ on the gel and roll up, lifting the gel off the plate.
29. Rinse the plates with water to remove any remaining pieces of gel. Wash with a detergent such as Micro Cleaning Solution that will not leave a residue, rinse with deionized water, and dry with a lint free towel. Rinse the comb and spacers with water.

Sequencing Data Analysis via Software

Follow instructions and software provided by automated sequencing instrument manufacturer to analyze electropherogram sequencing data and to assign HLA alleles. For the PE-ABI 377 sequencer, this procedure requires several steps briefly described here:

1. Review the tracking of the gel, assigning appropriate lanes to each sample sequenced.
2. Ensure that the data is saved and that backup copies are made.
3. Use sequence editor software to inspect the electropherogram, making corrections to initial nucleotide calls made by the instrument software. Carefully inspect potentially heterozygous positions.
4. Trim sequence data which is not necessary for or which complicates allele calling, e.g., intronic sequences.
5. Use manufacturer software to compare sequences obtained for samples with libraries of known allele sequences to identify the amplicon sequences.
6. Print paper copies of electropherograms and software analysis if desired for case review.

Procedure Notes and Limitations of the Procedure

1. Unambiguous allele identification is dependent on inclusion of all relevant polymorphic positions within the PCR template for sequencing. For example, if a crucial polymorphism in exon 4 uniquely defines an allele, a PCR template from exons 2 and 3 will not allow precise allele identification.
2. The detection of heterozygous positions on electropherograms is critically dependent on equal amplification of the two alleles present at a Class I locus in the PCR. PCR primers must be chosen carefully and empirically validated using a wide variety of allele combinations to guard against allele "drop-out" and inappropriate calls of homozygosity.
3. The Class I loci have many possible allele combinations which can result in ambiguous typings even when the complete sequences of exons 2 and 3 are determined. These so called ambiguous heterozygous allele combinations result from the inability to know whether a heterozygosity at one position in a sequence is in *cis* or *trans* linkage with a second heterozygosity elsewhere in the sequence. These difficult heterozygous combinations can be solved with allele specific PCR in the preparation of sequencing templates so that hemizygous electropherograms containing the sequence of only 1 of the 2 alleles present can be assessed.
4. Direct sequencing is generally not as sensitive to contamination of samples with genomic DNA or PCR products from other patients as other methods such as sequence specific oligonucleotide probe hybridization. This is both an advantage and a limitation. Laboratories should be aware that contamination might be present but not detectable in an electropherogram.

References

1. Blasczyk R, Wehling J, Kotsch K, Salama A. The diversity of the HLA class I introns reflects the serological relationship of the coding regions. Beitrage Zur Infusionstherapie und Transfusionsmedizin. 1997, 34:231-5.
2. Cereb N, Maye P, et al. Locus-specific amplication of HLA class I genes from genomic DNA: Locus- specific sequences in the first and third introns of HLA-A, B, and C alleles. Tissue Antigens 1995, 45: 1-11.
3. Iwanaga KK, Eberle M, Kolman CJ, Bermingham E, Watkins DI. Further diversification of the HLA-B locus in Central American Amerindians: new B*39 and B*51 alleles in the Kuna of Panama. Tissue Antigens 1997, 50:251-7.
4. Lee KW, Steiner N, Hurley CK. Clarification of HLA-B serologically ambiguous types by automated DNA sequencing. Tissue Antigens 1998, 51:536-40.
5. Petersdorf EW and Hansen JA. A comprehensive approach for typing the alleles of the HLA-B locus by automated sequencing. Tissue Antigens 1995, 46:73-85.
6. Sanger F, Nicklen S, Coulson AR: DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 1977, 74:5463-5467.
7. Scheltinga SA, Johnston-Dow LA, White CB, van der Zwan AW, Bakema JE, Rozemuller EH, van den Tweel JG, Kronick MN. Tilanus MG. A generic sequencing based typing approach for the identification of HLA-A diversity. Human Immunology 1997, 57:120-8.
8. Turner S, Ellexson ME, Hickman HD, Sidebottom DA, Fernandez-Vina M, Confer DL, Hildebrand WH. Sequence-based typing provides a new look at HLA-C diversity. Journal of Immunology 1998, 161:1406-13.
9. van der Vlies S, Voorter CE, van den Berg-Loonen EM. A reliable and efficient high resolution typing method for HLA-C using sequence-based typing. Tissue Antigens 1998, 52:558-68.

HLA-DR Sequence-Based Typing

Lee Ann Baxter-Lowe

Principle/Purpose

This procedure describes the use of automated nucleotide sequencing to determine HLA-DR types. The procedure described here has been used with a PE Biosystems 377 PRISM 377™ DNA Sequencer. There are several components to this procedure: selective amplification of target alleles, agarose gel electrophoresis to assess the quantity and quality of amplicons (PCR products), purification of amplicons, cycle sequencing reactions using ABI PRISM d-Rhodamine Terminator Cycle Sequencing Ready Reaction Kit™, purification of cycle sequencing products, electrophoresis using an PE Biosystems 377 PRISM 377 sequencer, and interpretation of the data. This procedure reliably produces high quality sequencing data (strong signal, low background noise). One disadvantage of this approach is that large PCR reactions are required to generate sufficient material to perform the purification steps. The rationale for methods used in this procedure along with suggestions for some alternative methods are provided below. Each laboratory must select components that are optimal for that site taking into consideration factors such as available equipment, test volume, cost, and turn-around-time. This procedure can be applied to HLA-DQB using PCR primers and conditions described by Voorter et al.

1. The PCR is used to generate templates for the sequencing reactions (primers described in Table 1). Genomic DNA (isolated using a QIAamp™ DNA Blood Minikit) serves as a template for selective amplification of individual alleles or groups of alleles. The amplicons (PCR products) are subsequently used as templates for cycle sequencing reactions. Selective amplification of single alleles is desirable because the sequencing data are easier to interpret and the only possible ambiguities (i.e., multiple interpretations) involve a small number of polymorphic sequences located outside the sequenced region. Use of templates with more than two alleles (e.g., a combination of two DRB1 alleles plus one or more DRB3/4/5 alleles) is not recommended because it is very difficult to accurately interpret sequencing data from three or more templates. Alternative sources of templates include RNA (RT-PCR) or cloned DNA.
2. The specificity and quantity of sequencing template influence the reliability and quality of sequencing data. The specificity and quantity of the amplicons are routinely assessed using agarose gel electrophoresis. Ideally, the PCR product migrates as a single, strongly staining band of correct size. The presence of multiple bands is sometimes indicative of loss of specificity. If the PCR is inefficient, a weak band is observed. Sometimes it is possible to compensate for inefficient PCR by making adjustments in subsequent purification steps and/or increasing the volume of DNA added to the cycle sequencing reactions.
3. Excess primer and unincorporated nucleotides can have substantial adverse effects on sequencing reactions (related to priming from the two PCR primers and/or altering the concentration of nucleotides). Optimal sequencing data are obtained if the amplicons are purified before use as templates in cycle sequencing reactions. This procedure used a commercial kit (HighPure™, Boehringer Mannheim) to purify the template by specific binding of PCR products to glass fibers in the presence of a chaotropic salt. There are several alternative methods for purification of PCR products including columns for purification (e.g., QIAquick™ PCR Purification Kit or Centricon 100™ columns) or enzymatic removal of excess nucleotides and primers. Werle et al. and Hanke and Winke described use of exonuclease I to degrade excess primers along with shrimp alkaline phosphatase to dephosphorylate residual nucleotides. This method is easy, relatively inexpensive, and amenable to high throughput. If the PCR reactions are very efficient with minimal primer and nucleotides remaining after the PCR, it is possible to dilute the template (typically 1:5 to 1:10) for direct use in sequencing reactions. If PCR efficiency is variable (e.g., due to suboptimal quality and/or quantity of template) or the substantial quantities of unincorporated primer and/or nucleotides remain in the PCR mixture, direct dilution is not recommended.
4. Purified PCR products serve as templates for cycle sequencing reactions using an ABI PRISM d-Rhodamine Terminator Cycle Sequencing Ready Reaction Kit™. Sequencing reactions contain modified nucleotides (dideoxynucleotides) that terminate polymerization when they are incorporated into the replicating strand of DNA. These terminated products are separated on the sequencing gel and the automated sequencer detects dyes that are incorporated into the products of the cycle sequencing reactions. Dye is introduced via a dye-labeled primer (dye primer chemistry) or dye-labeled dideoxynucleotide terminator (dye terminator chemistry). Custom dye primers can be purchased in kits or obtained by custom synthesis (expensive). Another alternative is to use PCR primers that contain a tail, which can be hybridized to a labeled primer.
   Disadvantages of the dye primer chemistry approach include detection of premature termination products that cause substantial problems during interpretation of the sequencing data and the technique's cumbersome set up, i.e., four reactions/sequence. However, using the dye-labeled dideoxyterminator procedure, which is performed in a single tube and are insensitive to premature termination products, eliminates these problems. The reason premature termination products are not detected is that they not labeled and, thus, transparent to the sequencer.

Another disadvantage of the early dye-labeled primer chemistry approach is variable peak heights caused by enzymatic differences in nucleotide incorporation. Variability in nucleotide incorporation has been minimized by development of enzymes that reduce discrimination against dideoxynucleotides (e.g., AmpliTaq™, FS, which has a point mutation in the active site). The chemistry of the dye molecules has also been improved over time to minimize differences in relative migration of molecules containing the dyes and spectral overlap.

5. A major disadvantage of the dye-terminator method is that the sequencing signals can be significantly decreased or totally obscured by the presence of dye-labeled nucleotides remaining from the cycle sequencing reactions (i.e., failed sequences in which all or part of the sequence is not interpretable). For this procedure, reliable and complete removal of excess labeled nucleotides is achieved using commercial spin columns. For methods that have a low quantity of unincorporated dye-labeled nucleotide after the cycle sequencing reaction, an inexpensive and quick alternative method for removal of excess nucleotides is ethanol precipitation. For methods that have large amounts of unincorporated dye-labeled material an alternative purification method is organic extraction.
6. After purification with spin columns, cycle sequencing products are dried and resuspended in loading buffer. Samples are denatured by incubating at 96°C for 2 min., quickly cooled on ice, and immediately loaded onto a sequencing gel. The sequencer must have run modules and mobility files that are appropriate for d-Rhodamine chemistry (files available at http://www2.perkin-elmer.com:80/ab/abww0008.htm).
7. The data are analyzed using ABI software to assign nucleotides, compare the unknown sequence to a sequence library, and to identify alleles that are identical or similar (up to 3 base pair differences) to the unknown sequence. Base identification is manually checked for accuracy and the type(s) are assigned. Factors that are considered in the analysis of the data include quality of the data (e.g., signal to noise ratio, signal strength, and spacing), number of unassigned nucleotides, number of discrepancies with constant (non-polymorphic nucleotides), and ambiguities (multiple interpretations of the data).

Specimen

Genomic DNA, (equivalent to DNA isolated using a QIAamp DNA Blood Minikit), 0.1 to 0.7 µg DNA / 100 µl reaction recommended

Unacceptable specimens include specimens with highly degraded DNA, specimens containing inhibitors of PCR, and specimens with concentrations of DNA that are either too low or too high to achieve efficient amplification. The quantity of DNA can be measured using A260 and A280 (see Section V.D.1.1, this manual) and the quality of DNA can be assessed by running an agarose gel to examine the size of the fragments (V.D.1.1).

If low resolution types are known, primers can be selected to amplify the groups of alleles that are in the specimen. If low resolution types are unknown, the entire panel of PCR primers for the locus is used.

Reagents and Supplies

General

Microcentrifuge tubes
Disposable gloves
Tips for micropipettors
Microcentrifuge tube racks
Vortex mixer

Preparation of Templates for Cycle Sequencing

Tubes for thermal cycler
PCR Buffer(s) (Table 1)
Nucleotide premix (2.5mM each dATP, dCTP, dGTP, dTTP, pH 7.0)
AmpliTaq™ polymerase
Primers, 5.0µM (see Tables 2 and 3)

Agarose Gel Electrophoresis

2% Agarose gel (mixture of 1% NuSieve and 1% LE recommended)
TBE (10.8 g Tris base, 5.5 g boric acid, 4 ml 0.5M EDTA, pH 8.0, volume adjusted to 1 L)
Molecular weight standards appropriate for PCR products of about 300 base pairs
5X Loading buffer (0.25% [w/v] bromphenol blue, 0.25% [w/v] xylene cyanol FF, 30% glycerol [v/v])
Tubes or microtiter plate for preparing samples for loading
Ethidium bromide (10 mg/ml stock, dilute to 0.5 µg/ml for use in gel and running buffer or stain gel in 0.5 µg/ml ethidium bromide after completion of electrophoresis)

Purification of PCR Products

High Pure™ PCR Product Purification Kit (Boehringer Mannheim 1732668)

Cycle Sequencing
- d-Rhodamine Terminator Cycle Sequencing Ready Reaction with AmpliTaq™ DNA polymerase FS (PE Biosystems 403043)
- Sequencing Primer, 50µM (see Tables 2 and 3)
- Tubes for thermal cycler
- Sequencing standard (e.g., pGEM which is included in the sequencing kit or a well characterized HLA-DR template)

Purification of Cycle Sequencing Extension Products
- CENTRI-SEP™ columns (Princeton Separations CS 901)

Sequencing Gel
- Long Ranger™ Single Pack (FMC, 50691)
- 10X TBE (108 g Tris Base, 55 g Boric Acid, 7.44 g Na_2EDTA 2-H_2O, QS to1L with dH_2O
- Blue dextran-EDTA sample loading buffer
- Deionized formamide, 200 µl aliquot, stored at -20°C
- 60 cc syringe (optional, to clean wells of gel)

Instrumentation/Special Equipment

General
- Micropipettors (2 µl, 10 µl, 20 µl, 100 µl, 200 µl)
- Vortex
- Vacuum aspiration apparatus (Speed Vac)

PCR Preparation of Templates for Cycle Sequencing
- PE Biosystems Model 9600 Thermal cycler (appropriate thermal cycling conditions must be empirically determined for other models)
- Microcentrifuge
- Heating block for denaturation

Agarose Gel Electrophoresis
- Electrophoresis chamber
- Power supply

Purification of PCR Products
- Centrifuge for High Pure columns (13,000 x g)

Cycle Sequencing
- Thermal cycler
- PCR hood or other biocontainment hood (recommended)
- Heating block for denaturation

CENTRI-SEP™ Purification of Cycle Sequencing Extension Products
- Microcentrifuge

Sequencing
- ABI 377 PRISM 377™ DNA Sequencer
- Heat block, preheated to 95°C
- Insulated container with ice
- MacIntosh or other computer equipped with software for sequence analysis, graphic presentation of data, and assignment of HLA types

Calibration

The sequencer must have run modules and mobility files that are appropriate for this type of chemistry (files available at http://www2.perkin-elmer.com:80/ab/abww0008.htm).

Quality Control

For agarose gels, the molecular weight markers must migrate and stain according to defined criteria. Deviations from the expected results may indicate technical problems with electrophoresis or staining that invalidate evaluation of unknown bands cannot be appropriately assessed.

According to current ASHI standards, a sequencing standard must be run on every gel. This could be the pGEM control provided in the d-Rhodamine Terminator Cycle Sequencing Ready Reaction kit or a local HLA template. The determined sequence should be identical to the expected sequence. Deviations from the expected sequence must be examined to identify the cause of the problem and to determine the acceptability of other sequences determined on the same gel.

Each laboratory must establish criteria for acceptance of each gel and each lane of a gel. Accurate assignment of bases located in non-polymorphic positions (constant positions) is one criteria that can be used for QC. If a base assignment for a non-polymorphic position is different from the expected nucleotide, the sequencing data be examined to determine if a novel polymorphism or technical problem exists. Other criteria for acceptance of sequencing data include spacing (spacing between 8 and 16 is acceptable, spacing of -12 can indicate a problem), signal numbers (signal strengths of dyes representing each base), and background (presence of nonspecific signals). Data should be rejected if the spacing is outside the acceptable range and there is any indication that the base calls could be incorrect. Data should be rejected if signal and/or noise make it difficult to clearly assign each nucleotide.

Procedure

1. Determine the primers that are required for the sample. If low resolution HLA-DR types are known, select the appropriate primer pairs to selectively amplify the alleles in the sample. If there are two alleles amplifying in a amplification group (i.e., same first hyperpolymorphic region) and codon 86 is different in each of the alleles, the alleles can be selectively amplified using the codon 86 primers. If low resolution HLA-DR types are unknown, the entire panel can be used for PCR and those reactions generating PCR products are used for sequencing.
2. Preheat thermal cycler to 98°C
3. Place tubes on ice and add the following reagents to each tube:
   63 µl Reagent grade water
   10 µl 10X PCR buffer with appropriate [Mg]
   4 µl Primer 1 (5 µM)
   4 µl Primer 2 (5 µM)
   8 µl nucleotide premix
   1 µl AmpliTaq™ DNA polymerase (1-5 units/ µl)
   10 µl genomic DNA
   Perform this step as quickly as possible.
4. Immediately begin thermal cycling
   Place tubes in thermal cycler
   Perform thermal cycling as follows:
   Rapid thermal ramp to 96°C
   96°C for 30 sec
   Rapid thermal ramp to 60°C
   60°C for 60 sec
   Rapid thermal ramp to 72°C
   72°C for 105 sec
   Repeat for 30 cycles
   Hold at 4°C
5. Run an agarose gel to evaluate the quantity and quality of amplicons. Add 2 µl loading dye to 8 µl amplicon. Stain with ethidium bromide. Prepare a record of the stained DNA using a camera or imaging device. A single, strongly staining band of appropriate size should be present. If the intensity of staining is weak, but a single band is present, sequencing may be successful if the concentration of the template is increased by decreasing the elution volume at step xx. If multiple bands are present, the quality of the sequence data may be unacceptable.
6. Purify each acceptable amplicon using High Pure™ PCR Product Purification Kit according to the manufacturers instructions, except that the purified product is eluted in 90 µl elution buffer. If the intensity of the band is weak, reduce the quantity of elution buffer in proportion to the relative intensities of the bands. The minimum recommended volume is 45 µl. Recovery is approximately 80% for specimens containing 25 µg DNA and eluted in 100-200 µl. Reducing the DNA concentration or elution volume lowers the recovery. Reducing the elution volume increases the concentration of DNA.
7. Set up cycle sequencing reactions by adding the following to each thermal cycler tube:
   8 µl d-Rhodamine Terminator Cycle Sequencing Ready Reaction mix
   5 µl water
   6 µl purified DNA template
   1 µl primer (5 µM)
   20 µl total volume
   Note: If the quantity of PCR product is low, the quantity of DNA can be increased with corresponding reductions in water. The fluorescent dyes are light sensitive. Whenever possible keep samples containing fluorescent dyes in the dark.

8. Perform thermal cycling.
   Rapid thermal ramp to 96°C
   96°C for 10 sec
   Rapid thermal ramp to 50°C
   50°C for 5 sec
   Rapid thermal ramp to 60°C
   96°C for 4 min
   Repeat for 25 cycles
   Hold at 4°C
9. Use CENTRI-SEP columns to purify cycle sequencing products to remove excess dye terminators. Place a mark on the outside of each column to use for aligning the columns in the centrifuge. Use columns as recommended by the manufacturer. The columns can be reused (up to five times) by removing the gel and adding 950 µl of a 7.5% Sephadex (G50 fine) slurry. Because reused columns sometimes become defective, a column should be discarded if the sequencing data from the most recent purification are consistent with column failure (e.g., terminator blobs, high background). Whenever possible keep samples containing fluorescent dyes in the dark.
10. Prepare and run the sequencing gel following the instructions of the manufacturer.
11. Analyze data according to the manufacturers instructions. The electropherogram should be manually reviewed to confirm base assignments. If the template is potentially heterozygous, it is particularly important to check for potential heterozygous bases. In some cases one peak may be too small to reach the threshold for automated assignment of heterozygous positions. MatchTools or MatchMaker can be used to compare the determined sequence to a library of all known sequences. The program identifies all perfect matches as well as those that are identical with the exception of 1, 2, or 3 differences.

Calculations

N/A

Results

Accurate sequences (100% accuracy) are obtained.

Procedure Notes

Since HLA is extremely polymorphic, it is essential to achieve 100% accuracy in the interpretation of data. Sequencing of the complementary strands of each template is recommended to ensure this level of accuracy. If the sequence of only one is determined (e.g., to reduce costs), it is necessary to validate the method using many polymorphic sequences (preferably one example of each polymorphic motif in various combinations) to ensure that artifacts do not occur. Occasionally there is minimal or no incorporation of a particular nucleotide. This type of artifact can be influenced by the combination of alleles in a sample.

Rarely, a particular HLA-DR allele may not amplify because denaturation fails. This may be related to proximity to regions of very high GC content which serve as clamps during denaturation. If this occurs, the allele will usually amplify after shearing the DNA and/or boiling the DNA for 3 min and placing the sample immediately on ice.

Limitations

Ambiguous sequences (multiple possible alleles or combinations of alleles for the assigned sequence) sometimes occur because two or more alleles have identical sequences for the segment of the gene that is determined (i.e., the differences are outside the sequenced region) or because there are two alleles present and the composite sequence is identical for more than one combination. These can be resolved by sequencing the products of a more selective amplification or using an additional method (e.g., SSP) to resolve the ambiguity.

References

1. Hanke M, Wink M, Direct DNA sequencing of PCRamplified vector inserts following enzymatic degradation of primer and dNTPs Biotechniques 17(5):85860, 1994 (published erratum appears in Biotechniques 18:636, 1995).
2. PE Applied Biosystems, ABI PRISM™ d-Rhodamine Terminator Cycle Sequencing Ready Reaction Kit Protocol. 1997.
3. Perkin Elmer. Comparative PCR Sequencing. A Guide to Sequencing-Based Mutation Detection. The Perkin Elmer corporation, 1995.
4. Voorter CE, Kik MC, van den BergLoonen EM, Highresolution HLA typing for the DQB1 gene by sequencebased typing. Tissue Antigens 51:807, 1998.
5. Voorter CE, Rozemuller EH, de BruynGeraets D, van der Zwan AW, Tilanus MG, van den BergLoonen EM, Comparison of DRB sequencebased typing using different strategies. Tissue Antigens 49(5):4716, 1997.
6. Werle E, Schneider C, Renner M, Volker M, Fiehn W, Convenient singlestep, one tube purification of PCR products for direct sequencing. Nucleic Acids Res 22:43545, 1994.

Table 1.

SPECIFICITY	SEQUENCE	ORIENTATION/LOCATION (CODON)4
Generic DR	CGC CGC TGC ACT GTG AAG CTC TC	3′ / 87-93
DRB1-1	TG TGG CAG CTT AAG TTT GAA	5′/ 8-14
DRB1-15/16	C CTG TGG CAG CCT AAG AGG G	5′ / 7-14
DRB1-3/11/13/14	TTC TTG GAG TAC TCT ACG TCT	5′ / 7-13
DRB1-4	GT TTC TTG GAG CAG GTT AAA C	5′ / 6-13
DRB1-7	C CTG TGG CAG GGT AAG TAT A	5′ / 7-14
DRB1-8/12	G TAC TCT ACG GGT GAG TGT TAT TTC	5′ / 7-16
DRB1-9	T TTC TTG AAG CAG GAT AAG TT	5′/6-13
DRB1-10	CCA CGT TTC TTG GAG GAG	5′/5-10
DR Codon 86 G	CT GCA CTG TGA AGC TCT CAC	3′ / 86-92
DR Codon 86 V	CT GCA CTG TGA AGC TCT CCA	3′ / 86-92
DRB3	GCA CGT TTC TTG GAG CTG C	5′ / 5-11
DRB4 exon 2	TC TTG GAG CAG GCT AAG TG	5′ / 7-13
DRB4 exon 3	CCT AAG GTG ACT GTG TAT CCT T	5′ / 97-104
DRB4 exon 3	GAG AGG GCT CAT CAT GCT TGG A	3′ / 177-184
DRB4 intron	ACG TTT CTC ATT CCT GTC TAA	5′ / INTRON
DRB4 intron	TTG GTT ATA GAT GTA TCT GAT	3′ / 28-34
DRB5	C TTG CAG CAG GAT AAG TAT G	5′ / 7-14

Stem Cell Engraftment Analysis Using PCR Amplification of VNTR/STR Loci

Anajane G. Smith and Chris McFarland

Principle

Human identity testing may be accomplished by the analysis of genomic polymorphisms such as variable number tandem repeat (VNTR) or short tandem repeat (STR) loci.[1-7] These loci consist of a core DNA sequence which is repeated a variable number of times within a discrete genetic locus. The terms VNTR and STR, also referred to as minisatellite or microsatellite DNA loci, relate to the number of base pairs of the tandemly repeated core DNA sequence. A VNTR, minisatellite, locus has a core of 8-50 base pairs,[8], while the core sequence of an STR, microsatellite, locus is 2-8 base pairs long.[9] Consequently, these loci exhibit alleles that may differ in length between individuals and are inherited as codominant Mendelian traits. VNTR and STR loci have been identified throughout the human genome and some loci have more than 25 alleles. With the availability of DNA sequence information on the conserved flanking regions of many VNTR/STR loci, oligonucleotide primer pairs have been synthesized to allow PCR amplification of these polymorphic loci. Since PCR amplification of a VNTR/STR locus is routinely performed with 10 ng of genomic DNA (equivalent to approximately 1,500 cells), chimerism testing by these methods can be successfully performed even for patients with graft failure, severe leukopenia, or from hematopoeitic cell subset fractions. We have used VNTR/STR analysis to evaluate the engraftment status of patients who have received a stem cell transplant, to confirm the genetic identity of putative identical twins, and to detect in-utero derived maternal cell engraftment among patients with Severe Combined Immunodeficiency Syndrome (SCIDS). We have found that PCR amplification and analysis of VNTR/STR loci provides a rapid and reliable method for the evaluation of engraftment status in the stem cell transplantation setting.

Specimen

Chimerism Test Sample Specifications

A. Pretransplant Patient and Donor Samples

1. Archived DNA
2. Archived PBL
3. Archived LCL
4. Fresh or frozen whole blood (1-20 ml).
5. If necessary, buccal cells, hair root, cultured bone marrow stroma or skin biopsy samples may be obtained after transplant.

B. Patient Post-transplant or SCIDS Test Samples

1. For “Routine” test samples.
   a. 1-3 ml bone marrow aspirate, anti-coagulated with EDTA or heparin.
   b. 1-20 ml whole blood anti-coagulated with EDTA, ACD, or heparin.
2. For “non-routine” test samples.
   a. 20-50 ml whole blood for samples with low white blood cell (WBC) counts (less than 1000 WBC/µl).
   b. 20-50 ml whole blood for T-cell enrichment/granulocyte fractions.
   c. Flow Cytometry sorted cell fractions containing at least 5,000 cells.

Reagents and Supplies

A. Consumable Supplies

Sterile 1.5 ml microfuge tubes
Sterile 0.2 ml PCR amplification tubes.
Pipette tips, sterile filtered, for P20/P200
Pipette tips, sterile filtered, for P1000

Pipette tips, P20 fine tip for loading polyacrylamide gels (Costar #4853)
6% TBE 10 well pre-cast polyacrylamide gels (NOVEX, EC6265)
6% TBE 15 well pre-cast polyacrylamide gels (NOVEX, EC62655)
Gel drying cellophane (NOVEX, NC380)
Polaroid film

B. Cell Processing and PCR Reagents

Ultrapure H_2O
10X Red Cell Lysis Buffer (Intermountain Scientific, C-5670-IL)
D1S80 primer set (10 pm/µl)
APOB primer set (10 pm/µl)
33.1 primer set (10 pm/µl)
33.6 primer set (10 pm/µl)
SE-33 primer set (10 pm/µl)
YNZ-22 primer set (10 pm/µl)
HumTho-1 primer set (10 pm/µl)
Multiplex-I primer set (Lifecodes # 172001)
10X PCR buffer (Perkin Elmer)
10 mM dNTPs (Perkin Elmer)
25 mM Magnesium Chloride
AmpliTaq Gold® (Perkin Elmer)
Positive control genomic DNA

C. Gel Electrophoresis and Detection Reagents

Ultrapure H_2O
Ethidium Bromide
GeneMate 10X TBE buffer (Intemountain Scientific #5555-40)
100 bp DNA ladder (Gensura #SL-100L)
Bromophenol Blue
Xylene Cyanol FF
15% Ficoll 400
Methanol
Acetic Acid
Nitric Acid
Silver Stain Plus kit (Bio-Rad catalogue #161-0449)
Gel-dry Drying Solution (Novex, LC4025)

D. Solutions Prepared In-house

1. **1X Red Cell Lysis Buffer (RCLB)**
   Dilute 100 ml of 10X RCLB up to 1 liter with ultrapure H_2O.
2. **Ethidium Bromide Stain (8 mg/ml)**
   Warning: Ethidium Bromide is a carcinogen and teratogen. Wear gloves and a mask when preparing this solution.
   Dissolve 80 mg ethidium bromide in 10 ml ultrapure H_2O.
3. **1X TBE Buffer**
   Dilute 400 ml of 10X TBE up to 4 liter with ultrapure H_2O.
4. **6X Ficoll loading buffer:**
   Bromophenol blue 0.25 g
   Xylene cyanol FF 0.25 g
   15% Ficoll 400 in 100 ml ultrapure H_2O.
5. **Destain solution**

Methanol	1600 ml
Acetic acid	400 ml
Ultrapure H_2O	2000 ml

6. **Silver Stain Fixative (Bio-Rad system)**

Methanol	100 ml
Acetic acid	20 ml
Fixative enhancer conc.	20 ml
Ultrapure H_2O	60 ml

7. Silver Stain (Bio-Rad system)

Silver Complex Solution	5 ml
Reduction Moderator Solution	5 ml
Image Development Reagent	5 ml
Development Accelerator Soln. (pre-warm to room temperature)	50 ml
Ultrapure H_2O	35 ml

8. Stop solution

Acetic acid	200 ml
Ultrapure H_2O	3800 ml

Instrumentation/ Equipment

DNA thermocycler (Perkin Elmer Cetus model 9600 or 9700)
Spectrophotometer
25 ml disposable pipettes
P20 pipetter dedicated for pre-amplification
P200 pipetter dedicated for pre-amplification
P1000 pipetter dedicated for pre-amplification
P20 pipetter dedicated for post-amplification
Thermocycler tube rack and base
96-well microtiter plate
PAGE mini-gel electrophoresis apparatus (NOVEX, EI9001)
Power supply for electrophoresis
Disassembly knife for pre-cast gel cassette
Gel staining tray
UV transilluminator
Polaroid copy camera apparatus
Gel drying racks (NOVEX, (NI2380)

Calibration

At the beginning of each week, a power-up diagnostic test is performed by plugging in the spectrophotometer. This initiates a self-test which is printed out, dated and filed.

P20, P200 and P1000 pipettors should be calibrated on a routine schedule (at least once a year is recommended)

Quality Control

A. PCR Standards

1. Ultrapure sterile water
   a. Negative PCR reagent control.
   b. After PCR and PAGE, this control sample should have no evidence of amplified DNA in the stained gel.
2. Standard human genomic DNA known to provide reliable amplification with all VNTR primer sets.
   a. Positive PCR reagent control.
   b. Store concentrated standard DNA at –20°C.
   c. Prepare monthly working aliquot of standard diluted to 0.5 ng/µl.
   d. Store working aliquot at 4°C.
   e. Examine the amplified standard lane for bands of the appropriate size.
3. Standards are amplified for all VNTR loci used in each chimerism assay.
4. Standards are electrophoresed on the same gels as the chimerism test samples and carried through gel staining.
5. If amplified fragments are detected in the negative control, PCR reagents should be discarded. If amplified fragments of the wrong size are detected in the positive control, PCR reagents should be discarded.

B. Electrophoresis Size Standard

1. 1-2 µl of a 100 bp DNA size ladder of known concentration is loaded in one lane of each gel.
2. Record the approximate size/location of the amplified fragments for each patient pre-transplant and donor specimen at initial testing.
3. In subsequent tests for a patient, compare the amplified fragments with recorded pre-transplant fragment sizes to verify sample identity.

Procedure

A. Sample Processing

1. Peripheral blood samples, WBC preparation.
   a. Place whole blood sample into a 50 ml Falcon tube, 1-25 ml per tube.
   b. Add 1X RCLB up to 40 ml total volume per 50 ml tube.
   c. Mix by inversion.
   d. Incubate at room temperature for 5-10 minutes.
   e. Centrifuge at 2,000 RPM for 10 minutes.
   f. Remove the supernate without disturbing the cell pellet.
   g. Resuspend the white cell pellet in the residual liquid.
   h. Examine the cell pellet for residual red cell contamination.
   i. If necessary, repeat red cell lysis up to a total of 3 cycles of lysis.
   j. Proceed to DNA isolation.
2. Bone marrow samples, WBC preparation.
   a. Place marrow sample in a 50 ml Falcon tube.
   b. Add 1X RCLB up to 40 ml total volume.
   c. Mix by inversion.
   d. Incubate at room temperature for 5-10 minutes.
   e. Centrifuge at 2,000 RPM for 10 minutes.
   f. Remove the supernate without disturbing the cell pellet.
   g. Resuspend the white cell pellet in the residual liquid.
   h. Proceed to DNA isolation.
3. More extensive cell processing to isolate particular white cell subsets may be required to address certain clinical situations.
   a. Lymphocyte and granulocyte fractions may be enriched by standard Ficoll density gradient centrifugation.
   b. Specific white cell subsets may be prepared by positive or negative selection with monoclonal antibody preparations.
   c. Flow cytometry may be used to prepare highly purified white blood cells of specific lineages.

B. Quantitation of genomic DNA samples for VNTR/STR amplification.

Note: In order to achieve reliable, robust, and reproducible amplification of VNTR/STR loci, it is important to quantitate the sample DNA and use the same standard amount of DNA (10 ng) in each PCR amplification. Several reliable methods are available for DNA quantitation. We have used UV spectrophotometry for this purpose.

1. Collect patient pretransplant, donor, and patient post transplant samples.
2. Quantitate sample DNA by UV spectrophotometry.
   a. Dilute DNA 1/20 in ultrapure H_2O (25 µl DNA + 475 µl H_2O).
   b. Blank the spectrophotometer using ultrapure H_2O.
   c. Load the cuvettes containing the diluted DNA samples into carrier.
   d. Read the OD_{260} and OD_{280} values.
      (1) The OD_{260} equals the concentration in the original DNA sample stock in mg/ml or µg/ul (e.g., an OD_{260} of 0.145 of a 1/20 dilution means that the original sample has a DNA concentration of 0.145 mg/ml (µg/µl).
      (2) The ratio of OD_{260}/OD_{280} measures the purity of the DNA and should be between 1.7 and 2.0 to obtain reliable, robust amplification.
   e. Record the OD_{260} values.
   f. Samples derived from very low cell count samples, such as FACS sorted cell subsets may be used for amplification without quantitation.
3. Calculate the dilutions needed for sample DNA so that 20 µl contain the 10 ng DNA required for PCR amplification (10 ng/20 µl = 0.5 ng/µl – see Calculations section of procedure for details).

C. PCR Amplification

1. Turn off UV light in the static hood, turn on the visible light source and cover the space with clean bench covers.
2. Prepare DNA sample dilutions
   a. Label a set of sterile 1.5 ml microfuges tubes.
   b. Pipet 1 ml ultrapure H_2O into each tube.
   c. Add the amount of DNA for each sample as calculated above (B.3).
3. Label a set of sterile 0.2 ml PCR tubes.
   a. Prepare one set of tubes for each locus/primer pair to be amplified.
   b. Number the tubes sequentially according to the samples and the locus amplified.
   c. Assign numbers for the negative (ultrapure H_2O) and postive (stock DNA) controls for each primer set.

4. Prepare a master mix for each locus/primer pair to be amplified as follows.

a. SE-33, D1S80, 33.6, ApoB, 33.1 individual locus amplification.

Reagent	*Quantity per tube*	*Final conc. In 50 µl*
10x PCR Buffer II	5.0 µl	1x
dATP (10mM)	1.0 µl	200 µM
dCTP (10mM)	1.0 µl	200 µM
dGTP (10mM)	1.0 µl	200 µM
DTTP (10mM)	1.0 µl	200 µM
$MgCl_2$ (25mM)	4.0 µl	2.0 mM
Amplitaq Gold Polymerase	0.5 µl	2.5 U/50 µl
3′ primer (10pm/ul stock)	1.25 µl	0.25 µM
5′ primer (10pm/ul stock)	1.25 µl	0.25 µM
Ultrapure H_20	14.0 µl	

b. HUMTHO-1, YNZ-22 individual locus amplification.

Reagent	*Quantity per tube*	*Final conc. In 50 µl*
10x PCR Buffer II	5.0 µl	1x
dATP (10mM)	1.0 µl	200 µM
dCTP (10mM)	1.0 µl	200 µM
dGTP (10mM)	1.0 µl	200 µM
dTTP (10mM)	1.0 µl	200 µM
$MgCl_2$ (25mM)	2.0 µl	1.0 mM
Amplitaq Gold Polymerase	0.5 µl	2.5 U/50 µl
3′ primer (10pm/ul stock)	2.5 µl	0.5 µM
5′ primer (10pm/ul stock)	2.5 µl	0.5 µM
Ultrapure H_20	13.5 µl	

c. Multiplex-1 amplification.

Reagent	*Quantity per tube*	*Final conc. In 50 ul*
10x PCR Buffer II	5.0 µl	1x
dATP (10mM)	1.0 µl	200 µM
dCTP (10mM)	1.0 µl	200 µM
dGTP (10mM)	1.0 µl	200 µM
dTTP (10mM)	1.0 µl	200 µM
$MgCl_2$ (25mM)	4.0 µl	2.0 mM
Amplitaq Gold Polymerase	0.5 µl	2.5 U/50 µl
Primer mix (Lifecodes)	5.0 µl	
Ultrapure H_20	11.5 µl	

2. Pipette 30 µl of master mix into each PCR tube.
3. Pipette 20 µl of prepared DNA sample dilution or control into a designated tube.
4. Cap each reaction tube and place cover on reaction tray base to transport to the thermocyclers located in the Post-amplification DNA Facility.

THE FOLLOWING PROCEDURES ARE PERFORMED IN THE POSTAMPLIFICATION DNA FACILITY USING DEDICATED POSTAMPLIFICATION EQUIPMENT, SUPPLIES AND REAGENTS.

5. Turn on the Thermocycler to allow heatblock cover to prewarm.
6. Remove the base from the PCR reaction tray and place the rack into the heatblock.
   a. Secure the PCR tube caps using the capping pen.
   b. Slide heatblock cover to close and turn knob until the cover is snug over the heatblock.
   c. Amplify all VNTR/STR loci with the following PCR profile:

Incubate	95°C	12 minutes (For activation of AmpliTaq Gold)
Denature	95°C	60 seconds
Anneal	60°C	45 seconds
Extend	72°C	60 seconds
	(30 cycles)	
Delay	72°C	10 minutes

7. Amplified DNA may be stored at 2-8°C up to one week before electrophoresis.

D. Polyacrylamide Gel Electrophoresis and ethidium bromide stain

1. Prepare the 6% TBE pre-cast gel for electrophoresis.
   a. Remove the comb and tape from the gel cassette.
   b. Use a transfer pipet to rinse the wells once with 1X running buffer.
   c. Displace bubbles from the wells leaving them full of running buffer.
2. Assemble the NOVEX gel electrophoresis apparatus and fill the "middle" chamber with sufficient FRESH 1X running buffer to cover the tops of the wells (about 150 ml).
3. Prepare samples for electrophoresis in a microtiter plate.
   a. Add 10 µl of amplified DNA to 2 µl of 6X ficoll loading buffer.
   b. Add 1-2 µl of a standard 100 bp DNA ladder and 8 µl of ultrapure H_2O to 2 µl of 6X ficoll buffer.
4. Underlayer the samples in their respective wells using P20 fine tips.
   a. Always include the 100 bp DNA ladder in one lane on each gel.
   b. Place sample buffer in the "empty" wells to promote uniform running of the stacking front.
5. Fill the outer buffer chamber with about 500 ml of 1X running buffer. (Buffer for the outer chamber may be reused once).
6. With the power off, make electrical connections to the gel tank.
7. Turn the power on and run at 100 V for the following times:

D1S80, 33.6	2.25 hours
SE-33, YNZ-22, Multiplex, HUMTHO-1	1.5 hours
ApoB	2.5 hours
33.1	3.0 hours

8. Turn off the power and disconnect the electrical leads.
9. Remove the gel cassette from the gel box.
   a. Pry the gel cassette open.
   b. Notch the upper left corner of the gel for orientation reference.
10. Remove the gel from the cassette into the ethidium bromide staining solution and incubate with agitation for 10 minutes.
11. Visualize and photograph the gel on a UV transilluminator.

WARNING:
ALWAYS WEAR A FACE SHIELD TO PROTECT FROM UV RADIATION WHEN USING THE TRANSILLUMINATOR

12. Destain the gel in 100 ml of destain solution.
    a. Replace with fresh solution three times over a one hour period.
    b. The gel may be left overnight in this solution.

E. Silver Stain and gel drying process

1. Drain the destain solution from the stain tub, add 200 ml of Silver Stain Fixative (Bio-Rad, see solution preparation section) and incubate at room temperature for 20 minutes with gentle agitation.
2. Drain the fixative and add approximately 200 ml ultrapure H_2O.
   a. Incubate for 10 minutes with gentle agitation.
   b. Repeat with another 200 ml ultrapure H_2O and 10 minute incubation.
3. Prepare fresh Silver Stain (Bio-Rad system).
   a. Pre-warm 50 ml Development Accelerator Solution to room temperature.
   b. Place 35 ml ultrapure H_2O into a beaker and stir with a Teflon coated stir bar. Add the following Bio-Rad reagents in the exact order:
      (1) 5 ml Silver Complex Solution.
      (2) 5 ml Reduction Moderator Solution.
      (3) 5 ml Image Development Reagent.
   c. Immediately prior to use, add 50 ml of the room temperature Development Accelerator Solution to the beaker.
   d. Drain the H_2O from the gel, add the silver stain solution to the gel and stain until the desired intensity is achieved.
4. Stop the staining reaction by draining the silver stain out and pouring approximately 200 ml Stop Solution (5% acetic acid) onto the gel and agitate gently for 15 minutes.
5. Rinse gel with ultrapure H_2O, changing with fresh water 2-3 times over a 10 minute interval.
6. Drain water and add Gel Drying Solution (100-200 ml) for 15 minutes.
7. For permanent data documentation, dry the silver stained gel with Novex gel drying supplies, as follows:
   a. Wet one cellophane in the gel drying solution.
   b. Set half of the blue drying rack with feet down on the mounting base.

c. Place wet cellophane on the rack.
d. Set gel on top of the cellophane and trim edges as needed.
e. Wet a second cellophane in gel drying solution and place over the gel.
f. Set the other half of the blue drying rack on top of the gel cellophane sandwich and clip the four sides together.
g. Set upright and dry overnight.

Calculations

DNA sample dilutions prior to PCR amplification to provide 10 ng of sample DNA in 20 µl volume (0.5 ng/µl).

1. Determine OD_{260} of a 1/20 dilution of sample DNA.
   0.5 / OD_{260} (of 1/20 dilution) = µl of DNA per 1 ml ultrapure H_2O
2. Use the following formula to determine the amount of sample DNA to add to 1 ml of sterile, ultrapure H_2O.

   0.5 / OD_{260} (of 1/20 dilution) = µl of DNA / 1000 µl ultrapure H_2O

   Example: for a sample with OD_{260} (1/20 dilution) = 0.100

   0.5 / 0.100 (OD_{260}) = 5 µl of original sample DNA is added to 1000 ul of sterile ultrapure H_2O

Results

A. Identification of Specific Markers

1. If a person is heterozygous at the VNTR locus, two bands will be seen.
2. If a person is homozygous at the VNTR locus, one band will be seen.
3. Examine the pre-transplant patient and donor samples at each locus amplified to identify at least one band (marker) specific to each patient and donor.
   a. Among related transplant pairs, analysis of 3 VNTR/STR loci provides both patient and donor specific markers in approximately 90% of cases.
   b. Among unrelated transplant pairs, analysis of 3 loci provides both patient and donor specific markers in over 95% of cases.
4. Sensitivity of detection is usually optimized by analyzing and evaluating markers that are close in size (less than 200 bp apart). This minimizes the effects of preferential amplification of lower molecular weight fragments.

B. Evaluation of Chimerism in the Test Specimens

1. Examine the patient post-transplant sample (samples) for the presence of the informative markers for patient and donor.
2. Determine whether the post-transplant sample is of host, donor or host plus donor (mixed chimerism) origin.
3. If the pre-transplant and donor samples appear the same on the first set of loci amplified (usually 3-4 different VNTR loci), repeat the test using additional loci.
4. In samples which exhibit mixed chimerism, comparison of band intensity between patient and donor specific markers allows semi-quantitative evaluation of the percent of patient and donor cells present in the original sample.

C. Test Sensitivity

1. Mixing experiments performed to optimize PCR conditions for each of the VNTR/STR loci listed below demonstrated that sensitivity of detection of a minority species is usually in the range of 1-5%, but may approach 0.1% under optimal conditions.
2. Test sensitivity declined when unique markers for recipient or donor were greater than 200 pb apart.
3. The SE-22, D1S80, 33.6, and ApoB genetic loci generally provide the most reliable and robust amplification with a high probability of identifying unique patient and donor markers and optimal test sensitivity.

D. Clinical Interpretation

1. The results of engraftment monitoring by the analysis of VNTR/STR loci in the stem cell transplant setting must always be evaluated in light of the clinical situation, the cell population being tested, the methods used to isolate each cell population tested, and the sensitivity of assay detection.
2. Close communication must be maintained between the clinical laboratories and the physicians who order chimerism tests. In this context, the distinction between testing for routine monitoring and testing for specific diagnostic purposes is extremely important.

Procedure Notes

A. DNA Isolation

The reliable isolation of high purity DNA is essential for PCR based chimerism testing. A wide variety of samples may be submitted for analysis depending on the clinical testing circumstances. Very low cell count samples may be encountered in patients early after transplant or in those with graft failure or severe leukopenia. Analysis of lineage separated sorted cell fractions comprised of as few as 5,000-10,000 cell may be required to answer particular clinical question. In addition we have found that reliable and robust PCR amplification of VNTR/STR loci with minimal production of potentially confounding background bands/fragments requires quantitation and use of a limited amount (10 ng) of high purity DNA.

B. Identification of Informative Markers

A major consideration in the identification of informative markers and the choice of loci for analysis is the potential for preferential amplification of lower molecular weight markers.[10] Maximum sensitivity for detection of patient cells is achieved when unique patient markers are located between or below the donor specific markers. Although the analysis of one locus can provide both unique recipient and donor markers, it may be desirable to analyze an additional locus to optimize assay sensitivity.

C. Reasons for Testing Genetic Markers in the Marrow Transplant Setting

1. Routine post-transplant documentation of host/donor origin of WBC.
2. Evaluate host/donor cells in patients with inadequate marrow function.
3. Define whether recurrent malignancy or EBV-lymphoproliferative syndrome has originated from host or donor cells.
4. Identify whether cells from a transfusion donor can be implicated in causing graft versus host disease.
5. Assess prognostic risks of rejection and recurrent malignancy.
6. Estimate the persistence of donor cells in patients with recurrent CML.
7. Evaluate whether rejection has occurred in:
   a. second transplant candidates.
   b. patients disorders at increased risk of rejection.
8. Verify genetic identity of twins.
9. Detect the presence of maternal derived cells in patients with SCIDS.

D. VNTR Primer Sequences

SE-33-3′	5′-AAT-CTG-GGC-GAC-AAG-AGT-GA-3′
SE-33-5′	5′-ACA-TCT-CCC-CTA-CCG-CTA-TA-3′
D1S80-3′	5′-GAA-ACT-GGC-CTC-CAA-ACA-CTG-CCC-GCC-G-3′
D1S80-5′	5′-GTC-TTG-TTG-GAG-ATG-CAC-GTG-CCC-CTT-GC-3′
APOB-3′	5′-CCT-TCT-CAC-TTG-GCA-AAT-AC-3′
APOB-5′	5′-ATG-GAA-ACG-GAG-AAA-TTA-TG-3′
33.6-3′	5′-AAA-GAC-CAC-AGA-GTG-AGG-AGC-3′
33.6-5′	5′-TGT-GAG-TAG-AGG-AGA-CCT-CAC-3′
YNZ-22-3′	5′-CAC-AGT-CTT-TAT-TCT-TCA-GCG-3′
YNZ-22-5′	5′-GGT-CGA-AGA-GTG-AAG-TGC-ACA-G-3′
33.1-3′	5′-TGC-TTT-CTC-CAC-GGA-TGG-GA-3′
33.1-5′	5′-CGT-GTC-ACC-CAC-AAG-CTT-CT-3′
HumTho-1-3′	5′-GTG-GGC-TGA-AAA-GCT-CCC-GAT-TAT-3′
HumTho-1-5′	5′-ATT-CAA-AGG-GTA-TCT-GGG-CTC-TGG-3′

E. VNTR Loci Descriptions

Locus (reference)	Chromosome	# of alleles	Allele size
D1S8O (1)	1	>29	250-650 bp
SE-33 (2)	6	>20	200-350
ApoB (3)	2	>12	550-900 bp
33.6, D1S111 (4)	1	>13	500-1000 bp
33.1 (4)	9	>10	400 bp-2Kb
YNZ-22 (5,6)	17	>11	200 bp- 2 Kb
HUMTHO-1 (7)	11	>8	180-200 bp
Multiplex-I D18S849	18	9	94-134 bp
Multiplex-I D3S1744	3	9	154-182 bp
Multiplex-I D12S1090	12	>25	212-306 bp

References

1. Budowle B, Chakraborty BR, Giusti AM, Eisenberg AJ, and Allen RC. Analysis of the VNTR locus D1S80 by the PCR followed by high resolution PAGE. Am. J. Hum. Genet. 1991: 48: 137-144.
2. Polymeropoulos MH, Rath DS, Xiao H, Merril C. Tetranucleotide repeat polymorphism at the human beta-actin related pseudogene H-beta-Ac-psi-2 (ACTBP2). Nucleic Acids Research 1992: 20: 1432.
3. Boerwinkle E, Xiong W, Fourest E, Chan L. Rapid typing of tandemly repeated hypervariable loci by the polymerase chain reaction: Application to the apolipoprotein B 3′ hypervariable region. PNAS USA 1989: 86: 212-216.
4. Ugozolli L, Yam P, Petz LD, Ferrara GB, Champlin RE, Forman SJ, Koyal D, and Wallace RB. Amplification by the polymerase chain reaction of hypervariable regions of the human genome for evaluation of chimerism after bone marrow transplantation. Blood. 1991: 77: 1607.
5. Wolff RK, Nakamura Y, and White R, Molecular characterization of spontaneously generated new allele at a VNTR locus: No exchange of flanking DNA sequence. Genomics 1988: 3: 347-351.
6. Horn GT, Richards B, Klinger KW. Amplification of a highly polymorphic VNTR segment by the polymerase chain reaction. Nucleic Acid Research 1989: 17: 2140.
7. Edwards A, Hammond HA, Jin L, Caskey CT, Chakraborty R. Genetic Variation at five trimeric and tetrameric tandem repeat loci in four human population groups. Genomics 1992: 12: 241-253.
8. Jeffreys AJ, Wilson V, Neuman R, Keyte J. Amplification of human minisatellites by the polymerase chain reaction: towards DNA fingerprinting of single cells. Nucleic Acids Res 1988: 16: 10953-10971.
9. Weber JL, May PE. Abundant class of DNA polymorphism which may be typed using the polymerase-chain reaction. Am J Hum Genet 1989: 44: 388-396.
10. Walsh PS, Erlich HA, Higuchi R. Preferential PCR amplification of alleles: mechanisms and solutions. PCR Methods and Applications 1992: 1: 241-250.

Analysis of HLA Alleles Using the TaqMan Method

William A. Rudert and Massimo Trucco

Principle/Purpose

High-resolution HLA class I and class II typing at the molecular level has become a routine laboratory assay, providing considerable information in such diverse areas as transplantation biology, population genetics and disease susceptibility. These advances in HLA molecular typing have been made possible by the development and application of a variety of techniques including restriction fragment length polymorphism (RFLP) analysis,[1-5] polymerase chain reaction (PCR) DNA amplification,[6,7] PCR-RFLP,[8-12] sequence-specific oligonucleotide probe (SSOP) hybridization,[13-15] "reverse" dot blot hybridization,[16-18] PCR amplification using sequence-specific primers (PCR-SSP),[19-21] and finally, direct sequencing.[22]

Serology, SSOP and SSP are the technical approaches most used for the detection of HLA Class I and Class II alleles. Each approach, however, has its own advantages and disadvantages. Current serologic methods, while highly specific, demand the use of nonrenewable pools of human antisera that must be continually tested and validated, and which, for the present time, may not adequately detect alleles associated with non-Caucasoid racial or ethnic groups. In fact, some of the phenotypic polymorphisms at certain loci within, for example, Oriental populations, are not revealed by the sera present in commercially available trays.[23,24]

The SSOP-based assay is, at present, widely used for the detection of HLA polymorphisms. While this assay is more feasible for testing large numbers of samples, it has the disadvantage of taking approximately four days from DNA extraction to allele designation. Laboratories employing SSOP analysis often report difficulties in assigning some alleles due to cross reactivity among the probes used. SSOP analysis is also limited in identifying certain heterozygous combinations that yield identical probe patterns with other heterozygous combinations.

The third type of approach described by a number of groups[19-21] uses sequence-specific primers (SSP) and PCR in a gel-based assay, in which the presence and length of the PCR product is the final readout. For example, Olerup and colleagues have described a set of SSP primers that defines 13 of the most common alleles at the DQB1 locus.[20] SSP has also been employed to identify alleles at the DRB1[19] and at the HLA Class I loci, both HLA-A[25,26] and HLA-B.[27,28] The specificity of PCR priming is augmented by inhibiting non-specific amplification through the introduction of a nucleotide mismatch near the 3′ end of the primer.[29] Although SSP strategy demands that a panel of PCR reactions be performed on each sample tested, it can offer higher resolution than SSOP alone. The number of specificities identified by this approach can continue to expand with the implementation of new or slightly modified primers, or by performing a "two-step" assay that incorporates a second round of PCR with additional primer sets.

The SSP approach offers several positive features: first, a DNA template can be used, thus obviating the requirement and expense of using viable cells as the targets of complement-mediated cytolysis or as the source of mRNA, both prerequisites for certain typing strategies. Second, the amplified fragments are short enough that they can be validated or confirmed by sequencing during the course of assay development. Third, from DNA extraction to allele identification, reliable and easily interpretable results can be generated in a few hours.[19,20] This short timeframe makes the SSP molecular alternative especially appealing for clinical HLA laboratories involved with cadaveric transplantation.

Although the SSP strategy is particularly well-suited for laboratories that analyze small numbers of samples, a gel-based strategy is less feasible for laboratories performing large-scale molecular typing. For this reason, it was necessary to design a molecular typing strategy that would combine the high-throughput advantage of SSOP with the speed, high resolution and relative ease of SSP analysis. Toward this end, SSP was used together with a modification of a recently described method that permits the amplification and direct detection of specific target DNA[30] with no requirement for post-amplification hybridization or gel analysis steps.

The new assay, sequence-specific priming and exonuclease-released fluorescence (SSPERF),[31] takes advantage of the 5′ to 3′ exonuclease activity of the Taq1 DNA polymerase normally used in PCR DNA amplification.[32] An oligonucleotide probe, labeled at the 5′ end with a "reporter" fluorescent dye and at the 3′ end with a "quencher" fluorescent dye, is added to each PCR reaction containing allele-specific primers. Internal control primers and a second doubly-labeled fluorescent probe containing a spectrally distinct 5′ reporter dye are also present in each PCR reaction. The annealing of either probe to its complementary PCR template strand during the course of amplification generates a substrate suitable for exonuclease attack. Cleavage of the hybridized probe generates smaller fragments that physically release the reporter from the quenching residue, enabling its detection by an increase in sample fluorescence (Fig. 1). Thus, the accumulation of specifically amplified DNA product is detected only under conditions in which the fluorogenic probe hybridizes to the amplified DNA and is enzymatically cleaved by the Taq 1 polymerase. The fluorescence signal is read directly from the PCR reaction mixture using a fluorescence spectrometer: e.g., the TaqMan.

Specimen

Cells and Cell Lines

A panel of homozygous cell lines such as those tested during International Histocompatibility Workshops, which cover all of the different alleles at the various loci should be used as the source of DNA to establish the method. DNA is prepared from these cell lines and/or from peripheral blood mononuclear cells obtained from unrelated individuals who carry rare alleles. The preferred method is one using a modified proteinase K and salt extraction protocol (see Section V.A.1 DNA Extraction Methods; also ref. 33) or QIAamp® Blood Kit (Qiagen, Santa Clarita, CA) following the vendor's guidelines.

Reagents

Sequence-Specific Primers and Probes

For the sake of clarity, the sequence-specific primers and doubly fluorescent probes at each locus will be considered independently.

Alleles at the HLA-A Locus

HLA-A locus-specific PCR reactions are generally performed using 34 sequence-specific primer pairs, a panel improved from that reported in the reference manual of the HLA class I SSP ARMS-PCR typing kit outlined in the 12th International Histocompatibility Workshop.[34] These primers are listed in Table I. To be properly used in this type of test, primer AL#37, the sense primer for reaction #3 (specific for A*02 alleles) must be substituted for the original sense primers described for reactions #28-32.[34,35] This substitution is necessary to permit hybridization of the probe to its complementary sequence between the sense and antisense primers. Also, the sequences of two of the primers, AL# C and AL# F, were modified by substituting an inosine (I) in the original sequence in order to further improve their specificities.[35] The new sequences are as follows:

AL#C' 5'-ggATgTAATCCTTgC I gTCgTAA-3'
AL#F' 5'-gCgCAggTCCT I TTCAA-3'

In the primer panel, the forward (i.e., sense) primers are specific for polymorphic sites in exon 2, while the reverse (i.e., antisense) primers are specific for critical sites in exon 3. These two exons encompass the great majority of Class I sequence polymorphic sites. Thus, the amplified segments span the region from exon 2 through exon 3, including an intron of 240bp in length.

Each amplification reaction also includes a pair of internal control primers, specific for the gene encoding the human *Adenomatous Polyposis Coli* (APC) protein, which amplifies a 251bp product[35].

PIC#I 5'-ATgATgTTgACCTTTCCAggg-3' (65°C)
PIC#A 5'-ATTgTgTAACTTTTCATCAgTTCg-3' (63°C)

This serves as an amplification control to verify sample integrity in the absence of a specific positive reaction. In addition, a negative control reaction containing distilled water in place of DNA should be included with each typing set. All primers can be synthesized using standard methods and an automated DNA synthesizer (e.g., 394 DNA/RNA synthesizer, Applied Biosystems Inc., Foster City, CA). These primers can also be purchased from LifeCodes, Inc. (Stamford, CT).

Four different fluorescent probes were devised for a comprehensive yet simple HLA-A typing strategy; three reveal the HLA-A specific amplifications, and the fourth probe recognizes APC gene amplimers. The probes were designed on the basis of published HLA-A sequences[36] and the APC gene sequence (GenBank accession nos. M74088 and M73548).

The 3 HLA-A-specific probes are labeled with the reporter dye FAM (6-carboxyfluorescein, PE Biosystems, Foster City, CA) at the 5' end. Added to the 3' end is an amino-modifier C6 dT linker (L) arm nucleotide (Glen Research, Sterling, VA) followed by a 3' phosphate (Phosphalink, Applied Biosystems, Foster City, CA) to prevent extension of the hybridized probe during PCR amplification. Other available dyes suitable for this labeling are the 4, 7, 2', 4', 7', 5'-hexachloro-6-carboxyfluorescein (HEX, PE Biosystems, Foster City, CA) or the tetrachloro-6-carboxyfluorescein (TET, also from PE Biosystems). These fluorescent reagents are diluted with acetonitrile and coupled to the extending oligonucleotides using conditions specified by the manufacturers. Following deprotection, the singly-labeled oligonucleotides are ethanol precipitated, resuspended in 0.25 M $NaHCO_3/Na_2CO_3$ (pH 9.0), and incubated overnight at 37°C with the quencher dye 6-carboxytetramethylrhodamine (TAMRA NHS ester, PE, Foster City, CA). Unreacted dye is removed by passage of the reaction mixture over a PD-10 Sephadex column (Pharmacia Biotech Inc., Piscataway, NJ). FAM-TAMRA, HEX-TAMRA and TET-TAMRA doubly-labeled probes are purified by reverse-phase HPLC using a DeltaPak C18 column (Waters, Bedford, MA) and an acetonitrile gradient that ranges from 10-80% acetonitrile in 0.1M triethyl ammonium acetate (pH 7.0). Individual fractions are collected, dried down, washed with ethanol, resuspended in sterile water and evaluated both for DNA concentration (by measuring the absorbance at 260 nm) and for low (<1) reporter-to-quencher ratios by measuring emission intensities at 518, 556 and 580 nm (the approximate maximum emission intensities for FAM, HEX and TAMRA, respectively) with the excitation wavelength set at 488 nm (see also "Calculations"). The internal control probe should contain at the 5' end as the reporter moiety a different fluorescent dye than that labeling the specific probe, but the same TAMRA dye at 3' end. The protocols for the synthesis and purification of fluorescent probes have been reported in detail elsewhere.[37] Otherwise, the same doubly-labeled fluorescent probes can be purchased as such from PE Biosystems, Foster City, CA.

The sequence, calculated annealing temperature and position for each fluorescent probe are:

HLA-AI 5′-FAM -CCCTgCgCggCTACTACAACCAgAgCgAgg-L-TAMRA-PO4- 3′, (82°C), (Exon 2: 311-340)
HLA-AII 5′-FAM-CgCTTCATCgCAgTgggCTACgTggACgAC-L-TAMRA-PO4- 3′, (82.6°C), (Exon 2: 133-162)
HLA-AIII 5′-FAM -CgCTCCgCTACTACAACCAgAgC-L-TAMRA-PO4- 3′, (68.9°C), (Exon 2: 314-336)
APC 5′ TET-AATCgAggTCAgCCTAAACCCATACTTCA-L-TAMRA-PO4- 3′, (82.0°C), (Exon 15)

"L" indicates the position of a linker arm °C6 dt, Glen Research, Sterling VA) which facilitates the coupling of the TAMRA dye. The 3′ phosphate prevents the probe from acting as a primer during PCR.

Although there are only 34 SSP mixes, certain primer pairs (specifically #9, #22, #25, and #26) identify a large set of alleles which can be subdivided by the use of the different HLA-A probes. Because only one HLA-A probe can be added in a PCR reaction mix (as all 3 HLA-A probes have FAM as the reporter dye), it is necessary to set up extra reactions with these primer pairs using different probes, generating a total of 38 reactions in a complete typing panel (Table I).

Alleles at the HLA-B Locus

For the B locus, 58 primer mixes must be used, together with one of 3 different doubly-labeled probes. The primers and corresponding probes are listed in Table II.

The sequence, calculated annealing temperature and position for each fluorescent probe are:

HLA-BI 5′-FAM-AACCTgCgCggCTACTACAACCAgAgCgAgg-L-TAMARA-PO4-3′ (86°C), (Exon 2: 237-267)
HLA-BII 5′-FAM-CgCTCCgCTACTACAACCAgAgC-L-TAMARA-PO4-3′ (72°C), (Exon 2: 241-263)
HLA-BIII 5′-FAM-CTCggACTCiTggCgTCgCTgTCgAA-L-TAMARA-PO4-3′ (82°C), (Exon 2: 105-130, antisense)

The control primers and probe are identical to the ones used for the HLA-A locus. Note also that HLA-BII and HLA-AIII are identical.

Alleles at the HLA-DR Locus

The 5′ nuclease PCR assay used to type donor samples for alleles at the HLA-DRB loci, was tested and validated using 22 sequence-specific pairs of primers for the most common DRB alleles (Table III; ref. 19). Only one DRB-specific fluorescent probe is required to detect the DR alleles:[37]

DRBI 5′-FAM-CTTCgACAgCgACgTggIggAgT-L-TAMRA-PO4-3′ (75°C), (Exon 2: 117-139)

Two amplification control primers C5 and C3 (19) specific for a region within the third intron of DRB1 are used in conjunction with the control probe:

C5 5′-TgCCAAgTggAgCACCCAA-3′ (60°C) (Exon 3: 234-252)
C3 5′-gCATCTTgCTCTgTgCAgAT-3′ (60°C) (Exon 4: 13-33)
DRCON 5′-TET-TCCCACATCCTATTTTCATTTCgCTCC A-L-TAMRA-PO4-3′ (78°C), (Intron 3)

Alleles at the HLA-DQ Locus

The primers specific for HLA-DQA1 and -DQB1 alleles are listed in Tables IV and V, respectively. One probe is sufficient for DQA1 allele detection, while three fluorescent probes (QB02, QB03 and QB05) were generated that differentially hybridize with DNA sequences internal to the sequence-specific primers used for DQB1-SSP (Figure 2). A fifth fluorescent probe, specific for a segment of the DRB1 third intron, was also generated to reveal amplification of the internal-control sequences in each PCR reaction. Each DQB1 probe is synthesized with the reporter dye 6-FAM at the 5′ end and the quencher dye TAMRA at the 3′ end. A different reporter dye, such as HEX or TET can be similarly attached to the 5′ end of the control probe. The sequences and melting temperatures of the fluorescent probes are as follows:

QB02 5′-FAM-CCgAgAAgAgATCgTgCgCTTCgACA-L-TAMRA-PO4-3′ (80°C) (99-124)
QB03 5′-FAM-CgAgAggAgTACgCACgCTTCgACAgC-L-TAMRA-PO4-3′ (81°C) (100-126)
QB05 5′-FAM-ATAACCgAgAggAgTACgTgCgCTTCgAC-L-TAMRA-PO4-3′ (80°C) (95-123)
DRCON 5′-HEX-TCCCACATCCTATTTTCATTTgCTCCA-L-TAMRA-PO4-3 (75°C) (Intron 3)

Probe QB02 reacts with DQB1*0201 and *0202; probe QB03 reacts with DQB1*0301, *0302, *0303 and *0304; and probe QB05 was designed to reveal hybridizations with alleles DQB1*0501, *0502, *0503, *0504,*0601, *0602, *0603, *0604, *0605 *0401 and *0402. The location of each probe relative to the DQB1 primers is shown in Fig. 2. The control probe (DRCON) hybridizes with a consensus sequence contained in the third intron of all DRB1 alleles. The HEX-labeled DRCON probe was the one originally described.[31] The TET-labeled used for the DRB locus[37] can be substituted and has proven to be a more stable reagent. All probes were designed with melting temperatures higher than those of the primers to ensure binding of the probes prior to primer extension during PCR.

The doubly-labeled fluorescent probe necessary to perform a HLA-DQA1 typing is as follows:

HLA-DQA 5 -FAM-TggACCTggAgAggAAggAgACTgCCT-L-TAMRA-PO4-3 (79°C) (110-136)

The control primers and control probe for HLA-DQA1 are the same as used for the HLA-DR locus. Due to the limited number of alleles present at this locus, DQA1 typing is quite simple to perform and analyze.

Note: PCR reactions specific for each locus have an optimal concentration of primers and probes, which are summarized in Table VI.

Supplies

Pipette-tips, ART®, Molecular Bio-Products;
MicroAmp™ reaction tubes (0.2 ml), PE Biosystems, Foster City, CA;
Disposable gloves, Pharmaseal #8877;
10X PCR Buffer, PE Biosystems, Foster City, CA, #N808-0153;
25 mM $MgCl_2$, PE Biosystems, Foster City, CA, #N808-0153;
10 mM Pre-mixed deoxynucleotide solutions, PCR Nucleotide Mix, Boehringer Mannheim, #1581295; store at -20°C;
Taq DNA polymerase, AmpliTaq®, PE Biosystems, #N808-0153;
Tissue culture grade water, Sigma;
15 ml conical centrifuge tubes (e.g., Falcon);
Plastic transfer pipettes (e.g., Samco);
1.5 ml microcentrifuge tubes (e.g., Fisher).

Instrumentation/Special Equipment

Automatic pipettes, capable of reliably dispensing 2 to 20, 50 to 200 and 100 to 1000µl (e.g., from Rainin)
394 DNA/RNA Synthesizer (PE Biosystems)
Programmable GeneAmp PCR System 9600 or 9700 Thermal Cycler (PE Biosystems)
TaqMan LS-50B (PE Biosystems)
Reverse-phase HPLC with a Delta-Pak C18 column (Waters Corporation, Bedford, MA)
Benchtop centrifuge (e.g., Sorvall RT6000B)
Variable speed microfuge (e.g., Beckman Microfuge 12)
Vacuum apparatus with aspirator
Water bath (56°C)
Rocking platform (e.g., Nutator)
Vortex (e.g., VWR Scientific)
UV sterile laminar flow hood

Calibration

Because the efficiency of TAMRA labeling and the purification of the double-labeled probes can vary from one preparation to the next, each new synthesis is checked using at least one of the following procedures.

ATP-dependent Nuclease Assay

Doubly-labeled probes are incubated for 4 hours at 37°C in the presence or absence of 13 units of ATP-dependent DNAse[38] (United States Biochemical, Cleveland, OH) at a final concentration of 0.5 µm, in a volume of 100 ml of buffer containing 20 mM $(NH_4)_2SO_4$, 5 mM $MgCl_2$, 0.5 mM ATP and 80 mM Tris-HCl, pH 8.9.[31] Following incubation, 4 ml of the digested and undigested probes are mixed and diluted to 40mL in PCR buffer to yield the following groups: 1) FAM/TAMRA probe (digested) plus HEX/TAMRA probe (undigested); 2) FAM/TAMRA probe (undigested) plus HEX/TAMRA probe (digested); 3) FAM/TAMRA probe (digested) plus HEX/TAMRA probe (digested). The probe mixtures are transferred into wells of a white 96-well microtiter plate (PE Biosystems, Foster City, CA) and scanned on a fluorescence spectrometer equipped with a plate reader (TaqMan LS50B, PE Biosystems, Foster City, CA). Fluorescence is measured at 518, 556 and 580nm as described above. Ratios of fluorescence intensity for FAM/TAMRA and HEX/TAMRA are calculated as described below.

Hairpin Assay

A "hairpin" oligonucleotide primer can be designed and synthesized for each probe, such that the nucleotides at the 3' end of the primer spontaneously form a perfect hairpin, the following two bases serve as a spacer and the remaining sequence is complementary to the target probe.[37] Formation of the hairpin and annealing with the probe in the presence of Taq DNA polymerase initiates probe cleavage (Figure 3). Pairs of the FAM- and TET-labeled probes are evaluated for multiplexing in PCR in the following combination: (i) absence of primers, (ii) presence of the FAM probe hairpin primer, (iii) presence of the TET probe hairpin primer, and (iv) presence of both primers. Probe-primer mixtures containing 0.25µM each hairpin primer are then subjected to a "mock" PCR under the same conditions used for target sequence detection and analyzed for fluorescence as described below.[37]

Quality Control

1. Internal Q.C. (Intra-Lab Comparison)
   a. DNA from known homozygous or heterozygous reference cells (controls) is amplified, hybridized, scored and analyzed as controls by each technologist weekly.
   b. The last sample from each run is repeated in the next successive run.
2. External Q.C. (Inter-Lab Comparison) – 20 samples per month provided, for example, by the National Marrow Donor Program.

3. Controls – Sequence Specific Primer (SSP) primer pairs are batch-tested against the same reference panel as used for SSOP.
4. Reagents-Basic Guidelines
   a. Do not open more than one container of a reagent or chemical at any one time unless the one in use is suspect.
   b. Note the date of any change of brand or method of preparation.
5. Reagents-Commercial Materials
   a. Upon receipt of supplies, mark the receiving and expiration date on the label before storage.
   b. When opened, label with date, technologist's initials, and expiration date.
6. Reagents-Prepared in the Laboratory
   Label properly (including working bottles) with:
   - Reagent name
   - Concentration and/or pH
   - Date of preparation
   - Technologist's initials
   - Date of expiration
7. Reagents-Water Utilization
   Tissue Culture Grade Water – purchased (Sigma, Cat.#W3500) and used for:
   a. DNA extraction
   b. Reconstitution of Primers and Probes.
   c. PCR reactions
   Type II Water – Nanopure Purification System (Barnstead)
   a. Making buffers and solutions in the Analytical Lab (post PCR).
   b. Water baths.
   c. Autoclaved aliquots are used in the DNA extraction protocol.
   Reverse Osmosis Water – (deionized Water) and used for:
   a. Glassware and plasticware rinsing.
   b. Filling water baths in the Analytical Lab (post PCR).
8. Oligonucleotides
   a. Procedure to store oligonucleotide primers. Specific primer mixes are prepared according to the combinations in Tables I-V at 20x the final reaction concentration. Each new primer set is tested against a panel of reference cells prior to use. The panel contains multiple examples of each allele to be detected (or not detected). Care must be taken to have new oligonucleotides synthesized and tested before the lot currently in use is depleted.
      (1) Oligonucleotide primers should be tested for their ability to amplify DNA with the appropriate specificity. Amplifications should be monitored by gel electrophoresis to detect ethidium bromide stained bands from the appropriate cells. Primer mixes are adjusted to 20x the final concentration, aliquoted and stored frozen, or in small working aliquots at 4°C. Control primer mixes are prepared at 50x the final concentration.
      (2) An aliquot of each mix should be tested for specificity against the reference panel.
      (3) Diluted oligonucleotides used as PCR primers may degenerate upon repeated freeze-thaw cycles. Individual oligonucleotide stocks are best stored lyophilized or in concentrated solutions. Repeated freezing and thawing of primer mixes is avoided by keeping aliquot sizes small enough to be used in a few days.
      (4) DNA probes degrade over time and lose specificity. Thus, it is important to monitor the specificity of the probes over time.
   b. Procedure to store oligonucleotide probes.
      (1) Dilute each probe to 50 pm/µl in Sigma tissue culture grade water.
      (2) Test each probe at appropriate amounts and document in an oligonucleotide log book. Aliquot 100 µl each into a sterile amber 0.6 ml vial. Store at -20°C.
      (3) For use, dilute to 1 pm/µl with tissue culture grade water. Use this working solution until you run out, or a problem with the probe reactivity is suspected.
9. Method for Performing Wipe Tests:
   NOTE: This procedure should be done weekly and should include a minimum of 10 samples, including such areas as lab benches, laminar flow hoods, work surfaces or centrifuges.
   a. Wear gloves and laboratory coat.
   b. Decontaminate forceps by wiping them with 10% bleach made with ddH_2O and then rinsing the forceps in ddH_2O.
   c. Wet a 1.0 cm diameter disk of Whatman 3 mm paper in ddH_2O using a pair of decontaminated forceps.
   d. Wipe wet filter paper over an approximate 10 cm square area.
   e. Place filter paper disk in a 1.5 ml microfuge tube with 400 µl of ddH_2O and briefly vortex.
   f. Incubate at 56°C for 1 hour.
   g. Quick spin to force the filter paper to the bottom of the tube.

h. Use 50 µl of the wipe test sample liquid in a 100 µl PCR reaction.
i. Amplify the wipe test samples using, for example, the DRB consensus primers following the lab's standard PCR protocol. Use the same number of amplification cycles as routinely used for donor samples.
j. Monitor DNA contamination by spotting the amplified material on a membrane and hybridize with DRB consensus probe. The membrane should also contain positive and negative controls.
k. If any areas are found to be contaminated, clean area thoroughly with 10% bleach, then retest. DNA prep area must test negatively before work can resume.

Instrument Care and Quality Control

NOTE: All temperatures are monitored for accuracy with calibrated alcohol thermometers independently of the instrument's internal temperature reading.

1. Laminar Flow Hoods: The top work areas are cleaned with 70% ethanol or 10% bleach solution after every use, followed by at least 20 minutes of UV irradiation The air flow is checked and serviced once a year by certified serviceman.
2. Refrigerators/freezers: Temperatures are recorded daily. Temperatures should be accurate within ± 2°C for refrigerators and ± 3°C for freezers.
3. Centrifuges: Centrifuges are cleaned after each use with 70% ethanol or 10% bleach solution.
4. Water baths: Temperatures of 37°C and 56°C water baths are recorded prior to each use. Water levels are checked daily. Shaking baths are cleaned monthly and other water baths are cleaned as needed. Temperatures of water baths should be accurate within ± 1°C.
5. Pipettors: Pipettors are checked for accuracy and reproducibility every 6 months. Each pipette is checked in triplicate. Use the pipette calibration worksheet to record pipette size, pipette identification number, and sample weights. Wear disposable gloves while obtaining weights. Pipettors are sent out for calibration and repair as needed.
6. Incubators: Temperatures of incubators are recorded before each use. Incubator temperature should be accurate within ± 1°C.
7. Thermal cyclers: Thermal cyclers are cleaned weekly with 50% ethanol and monthly with 10% bleach solution. Heater/chiller and verification of temperature calibration tests are performed monthly. Temperature accuracy and cycle time reproducibility tests are performed every six months. Consult thermal cycler user manual for protocols on performing the diagnostic tests.

Procedure

Polymerase Chain Reaction (PCR) Protocols

1. For each locus to be tested, pipette the set of specific primer mixes (2.5 µl each) and the corresponding specific fluorescent probe (1 µl) into an array of 0.2 ml and PCR tubes.[31,35]
2. Prepare a master mix sufficient for all the reactions, containing per each reaction:
   5 µl 1x PCR buffer
   5 µl pooled dNTPs (2 mM each)
   3.6 µl 25 mM $MgCl_2$
   3.6 µl 100 mM Tris (HLA-A locus ONLY)
   1.0 µl internal control primer mix
   1.0 µl control fluorescent probe
   0.25 µl Taq Polymerase (5 U/µl)
   250 ng DNA and H_2O sufficient to make 46.5 µl
   Except for the HLA-B locus, the 10x PCR buffer is 500 mM KCl, 10 mM Tris-Cl pH 8.3. The HLA-B 10x PCR buffer is 670 mM Tris-Cl pH 8.0, 166 mM Ammonium Sulfate, 10% Tween 20.
3. For each sample to be tested, prepare negative controls for each specific probe (1 µl) which also contain the appropriate internal control probe (1 µl) in 1x PCR buffer.
4. Pipette 46.5 µl of the master mix to all the tubes.
5. Perform PCR amplifications in a GeneAmp PCR System 9600 thermocycler using either two-step or three-step cycling parameter.
   a. Two-step reactions are as follows: 35 cycles of 95°C for 20 sec, 65°C for 1 min, followed by a final extension of 72°C for 5 min.
   b. Three-step reactions consist of 30 cycles of 96°C for 20 sec, 65°C for 30 sec and 72°C for 30 sec.
6. Transfer 40 µl of each PCR amplification mixture from the thermocycler tubes into wells of white 96-well microtiter plates (Perkin Elmer, Norwalk, CT);
7. Place 40 µl of 1x PCR buffer in one well to serve as a "blank";
8. Read with a fluorescence spectrometer equipped with a plate reader (TaqMan LS50B, PE Biosystems, Foster City, CA). In each experiment, 40 µl of the ATP-dependent DNAse digested and undigested probe mixtures should also be transferred to certain wells of the microtiter plate for comparison of fluorescence values. Fluorescence is measured at three wavelengths: 518, 556 and 580 nm.

Calculations

At each wavelength, the emission intensity of the PCR buffer blank is automatically subtracted from the intensities measured for each sample and control well. For each reaction mix, two ratios are calculated, for example: the first, RQ_{FAM}, is the fluorescence intensity at 518 divided by the fluorescence intensity at 580 (i.e., FAM/TAMRA), while the second, RQ_{HEX}, represents the fluorescence intensity at 556 divided by the fluorescence intensity at 580 (i.e., HEX/TAMRA). The fluorescence emission generated by TAMRA is unaffected by the presence or absence of amplification, and serves to normalize for well-to-well variation in probe concentration, pipetting errors or microtiter well inconsistencies. Finally, a value for ΔRQ is generated according to the following example for FAM fluorescence: RQ_{FAM} minus $RQ_{FAM(NT)}$, where $RQ_{FAM(NT)}$ is the FAM/TAMRA fluorescence ratio associated with the no-template controls.[31,35,37]

Evaluation of reporter dye quenching

In order to determine the extent of quenching of the reporter dye (i.e., FAM or HEX) by the TAMRA dye, it is useful to compare the reporter fluorescence of intact probes with that of probes that are maximally degraded by the activity of ATP-dependent DNAse.[31,35,37] This enzyme hydrolyzes nucleotides from both the 5′ and 3′ ends of linear double-stranded and single-stranded DNA, and requires ATP for its activity.[38] The efficiency of quenching can be estimated by comparing the emission intensities of the reporter dye in the intact and digested probes. Probes with quenching efficiencies that range from approximately 65% to 95% will work well in the assay. The fluorescence associated with the undigested and digested probe mixtures predicts the range of potential fluorescence of positive samples.

Results

Fluorescent Probe Specificity

Initial experiments are necessary to evaluate the exonuclease cleavage of several fluorescent probes during both generic – and SSP-PCR reactions using DNAs from homozygous cell lines.[31] In these experiments, the fluorescence signal in each reaction can be visualized by electrophoresis of an aliquot of each PCR reaction on polyacrylamide gels and analysis of the cleavage products using GENESCAN software. Under conditions of generic amplification using, for example, DQBampA and DQBampB primers,[39] the QB03 probe was cleaved regardless of whether the reaction contained template DNA from BM16 (*0301), WT51 (*0302), DKB (*0303), PGF (*0602) or EHM (*0501) cells; the negative control lacking template DNA did not exhibit probe cleavage. Thus cleavage of this fluorogenic probe was observed despite differences of up to two base mismatches between probe and template DNA. SSP amplifications also resulted in non-specific cleavage of a particular probe under conditions of one or two-base mismatches, but only if the "correct" primer pair was present in the reaction.

Results from a heterozygous sample (*0201/*0302) illustrate this point (Fig. 4). Sample #2300101 was analyzed using the fourteen DQB1 primer pairs listed in Table V, together with the appropriate fluorescent probe (i.e., either QB02, QB03 or QB05) in each reaction. Degradation of probes QB02 and QB03 occurred only in the presence of primer pairs B-5′07+ B-3′07 and B-5′08+B-3′08, respectively (Fig. 4). These results indicate that a positive fluorescence signal was dependent, as expected, upon both the specificity of the probe (which could anneal under conditions of up to two base mismatches), and the specificity of the primers within each reaction. The final combined "functional" specificity of both probes and primers therefore increased. This type of analysis can also be used to confirm that an SSP-based amplification strategy would require relatively few probes compared with a strategy using generic primers and a large panel of more specific doubly-labeled fluorescent probes.

Heterozygote Analysis

The ability of the SSPERF assay to detect the heterozygosity in DNAs prepared from peripheral blood lymphocytes should also be tested. Samples are subjected to SSP amplification with the appropriate primer pairs, control primers and fluorescent probes. In the case of DQB1, six patient samples plus all controls occupied a single 96-well plate; the experimental outline is shown in Fig. 5. The raw data from a typical experiment representing FAM, HEX and TAMRA fluorescence values for a single 96-well plate are given as an example in Fig. 5, and a confirmatory gel for these same samples is shown in Fig. 6. Both fluorescence and gel analyses confirmed the following typing of these six individuals: Patient #1: DQB1*0303/*0402; Patient #2: DQB1*0501/*0402; Patient #3: DQB1*0302 homozygote; Patient #4: DQB1*0501/*0201; Patient #5: DQB1*0603/*0302; Patient #6: DQB1*0603/*0201. Cross-reactive bands were occasionally observed, but did not generate positive fluorescence signals because of the specificity of the relevant probes in these reactions.

A band amplified by the *0602 primer pair was evident in the gel representing the typing for Patient #1 (Fig. 5, patient #1, lane 5). However, it did not hybridize with the QB05 probe used to detect authentic *0602 alleles; thus, no significant cleavage of the probe occurred (Fig. 5, well F1). Although the *0201/*0302 primer pair can amplify either of these alleles, the QB03 probe in this reaction mix efficiently hybridized to amplified *0302 sequences (see Fig. 6, Patients #3 and #5, lane 11 and Fig. 5 wells E9 and E11) but was not cleaved in the presence of an amplified *0201 allele (see Fig. 6, patients #4 and #6, lane 11 and Fig. 5, wells E10 and E12). The DRB1 control band was easily visualized in each reaction. The intensity of this band was often characteristically weaker than that of the specific band in positive specific reactions, due to PCR conditions that favor DQB1-specific amplifications by using lower concentrations of DRB1 primers relative to specific primers.

Figure 7 displays representative alleles detected by each of the three DQB1 fluorescent probes; the data represent the typing results obtained for 50 samples. An example of an allele that was not detected in this population (DQB1*0401) is included. The group of points that represents negative controls (i.e., probes alone) are tightly clustered around the origin of each graph. The negative amplifications for each indicated allele from all fifty samples are represented by another tight cluster of points that is shifted to the right in each graph due to the increased HEX fluorescence generated by the DRB1 positive control amplification in these samples. The ellipse around each cluster of data points reflects 5 standard deviations from the mean fluorescence generated by these DRB1 control amplifications. The minimal observed increase in (RQ_{HEX} fluorescence observed in these reactions versus the mean fluorescence of the negative controls also varied slightly and ranged from 4.7-8.5 standard deviations. The points depicting samples that were positive for the indicated allele appear along a diagonal whose slope approximates the increase in FAM fluorescence as measured at both the FAM and HEX emission maxima. These points fall within a range, indicated by the dotted lines that represent the fluorescence associated with increasing FAM/TAMRA cleavage in combination with either minimal or maximal cleavage of the HEX/TAMRA probe, as determined by ATP-dependent nuclease digestion. The difference in (RQ_{FAM} fluorescence between the weakest positive signal and the negative amplifications also varied from allele to allele and ranged from 8.6 to 74 standard deviations. Thus even a "weak positive" amplification resulted in sufficient cleavage of the hybridized probe to generate an enormous increase in FAM (DQB1-specific) fluorescence values that was easily separable from the fluorescence associated with amplifications of the DRB1 control alone.

Procedure Notes

Probe Design

The most critical element in the SSPERF assay is the synthesis of appropriate fluorogenic probes. Such probes must exhibit: 1) low background fluorescence (i.e., efficient quenching of the reporter dye fluorescence by the quenching dye); 2) efficient cleavage by Taq 1 polymerase between the nucleotides carrying the reporter and quencher dyes; 3) high specificity for the target DNA sequence; 4) compatibility with the temperature, $MgCl_2$ and cycling conditions optimal for SSP-amplifications; and 5) an inability to form hairpins, dimers or other significant secondary structures with the other primers or probes within the reaction.

The factors affecting reporter dye quenching are not completely predictable. The position of the quencher dye does not appear to influence the efficiency of quenching to a great degree; however, it has been reported that a G residue next to the reporter dye can impart a quenching effect that is independent of probe cleavage.[40] The choice of reporter and quencher dyes also potentially affects quenching, although we have not systematically compared probes synthesized with different combinations of fluorescent dyes. The degree of TAMRA labeling and the purity of the probe may affect probe performance because the presence of significant levels of singly-labeled oligonucleotides results in a poorly quenched probe and a high background reporter fluorescence. An ideal intact fluorogenic probe should exhibit approximately 90% quenching, although in practice, depending upon the probe sequence, this level of quenching is not always achievable. Every probe used was not quenched to this extent, but still worked well in this assay.

Factors that affect the efficiency of probe cleavage include the placement of the quenching dye relative to the reporter dye and the extent of hybridization of the probe to target DNA. While some investigators have placed the quencher dye internally,[41] others suggest that placing the quencher dye at the 3′ end both maximizes the potential to cleave the probe between the two dyes, and minimizes any likelihood that the dye will interfere with hybridization.[40] All of the probes described contain the reporter dye at the 5′ end and the quencher dye at the 3′ end of the oligonucleotide. Since most of the specificity of this assay comes from the choice of primers, probe design must conform to the constraints set by the primers themselves. As with conventional primer-probe combinations, adjustments in the length or in the choice of the particular sequence of a given probe are often sufficient to resolve additional problems associated with specificity, cycling parameters, secondary structure or incompatibility with other primers and probes.

Considering all of the criteria for appropriate probe design, it is desirable to choose an approach that would require as few fluorogenic probes as possible. For example, only three fluorescent probes can be used to detect all of the most common HLA-DQB1 alleles. To do this, they must be designed to absorb up to two-base mismatches. For HLA-A and HLA-B, one of the probes (HLA-AIII or HLA-BII) can be used for both loci. Analysis of other loci by this method requires even fewer probes: our experiments using the SSPERF strategy to detect 34 HLA-DRB1 alleles indicates that for this locus, only one DR-specific probe is required.[37] The number of control probes is also kept to a minimum.

Although the preparation and purification of fluorescent probes is more time-consuming than the simple synthesis and labeling of conventional fluorescent or radioactive probes for SSOP-based typing, such probes, once validated in the assay system, can be generated in large-scale (1-10μM) syntheses that will provide sufficient reagents for many thousands of reactions. For example, a single 1mM oligonucleotide synthesis would generate enough purified probe for approximately 200,000 reactions, taking into account the anticipated losses that are inevitable at each step in the synthesis and purification scheme. Once generated, the doubly-labeled probes can be aliquoted, together with the relevant SSP primer pairs, into appropriate master mixes and stored indefinitely at -20°C in PCR strip tubes.

Specificity

The use of SSP primers to generate allele or group-specific amplification products is a well documented and widely used method of increasing typing resolution, or clarifying "ambiguous alleles" flagged by SSOP. Adding fluorogenic probes to these reactions not only eliminate the requirement for a gel-based readout, but, in some cases, adds a second degree of specificity. For example, the primer pair B-5'08-B-3'08 originally described by Olerup et al.[20] amplifies both DQB1*0201 and *0302 alleles. The detection of both of these alleles in a conventional SSP assay demands two additional amplifications using B-5'07-B-3'07 primers (for the detection of the *0201 allele) and B-5'08-B-3'09 primers (for the detection of the *0302/*0303 alleles). In the described assay system, however, the QB03 probe discriminates between *0302 and *0201 alleles amplified by the modified primer pair B-5'08-B-3'08, and only the *0302 allele is detected. Similarly, the QB02 probe hybridizes only with *0201 target sequences and is used in combination with the B-5'07-B-3'07 primer pair to detect this allele. Thus the resolution of these two alleles is accomplished with only two amplification reactions, each using a different probe, rather than with three reactions as in conventional SSP. Eliminating this third reaction allows the inclusion of an additional primer pair for the detection of the *0605 allele (Table V), while limiting the total number of reactions to 14. This array permits the analysis of 6 DNA samples (including controls) on a single 96-well plate (see also Fig. 5).

Since the SSPERF assay detects the presence of specific amplification, but does not reveal any information regarding the size of the amplimer, it is important to validate the specificity of the SSP primers used before relying solely on the fluorescence readout. Obviously, primer pairs that give rise to non-specific amplification of other HLA alleles, which can still anneal to the included probe, will yield to false-positive results. However, the specificity of "problem" primers can often be enhanced by modifications such as those described for the B-3'08 and B-3'10 primers: the primer efficiency and specificity of these primers was improved by making the inosine substitutions detailed previously. Under our conditions, the modified B-3'10 primer, in combination with the B-5'08 primer, amplifies the DQB1*0303 allele in the absence of cross-reactive amplification of the DQB1*0302 allele. These primer modifications thus permit better discrimination between *0302 and *0303 alleles despite the use of a single probe (QB03) in both reactions. Taken together, these examples illustrate how the combined specificity of primers and probes increase the total functional specificity of an individual reaction beyond that of either component alone. A similar approach may be used to reduce the cross-amplification of *0303 by *0602-specific primer pairs, although this cross-reactivity does not interfere with the assay.

In general, there is complete concordance between the results obtained using SSPERF method and those using the conventional SSP method. In addition, even poorly amplified specific products, that are weak or are not easily visible in a gel, are readily detectable by the TaqMan assay. In some cases, SSPERF is able to subtype certain alleles not resolved by SSOP. Also, in some cases it is possible to assign homozygosity to samples that had been typed as potential heterozygotes by SSOP. In other samples, it is possible to reliably identify heterozygous typing where SSOP typing results are ambiguous.

Limitation of the Procedures

The fluorescence data summarized in Fig. 7 illustrate several points. Although the two reporter dyes, FAM and HEX, are spectrally distinct at 518 and 556 nm, there is still some spectral overlap between them. Thus, the signal generated by an increase in FAM fluorescence is detected primarily at the 518 wavelength, but is also observed to a lesser degree at the 556 (HEX) wavelength. These increases in apparent HEX fluorescence, due to FAM, can readily be observed in the raw data and translate graphically into the diagonal line that represents samples that are positive for the test allele. Although it would be possible to mathematically separate the fluorescent components into those independently contributed by the FAM and the HEX dyes, this degree of sophistication is unnecessary for this application. Since the HEX signal is informative only for the case of the negative amplifications of the test alleles, it is only necessary to accurately determine the HEX signal in the absence of a strong FAM signal.

The differences observed between high and low positives are likely due to sample-related excipient effects, such as trace salts or other impurities, that may affect probe hybridization and/or cleavage, as well as DNA amplification. However, it should be stressed that even the weakest positive sample generates a fluorescence signal that is almost 9 standard deviations higher than the average negative amplification. Of course, the threshold for what can be considered a positive sample depends upon the number of samples analyzed and the confidence limits one wishes to assign to the typing. It is wise to normally permit a sample to be called "positive" if its fluorescence value is at least 3 standard deviations above that demarcated by the ellipse around the control amplifications negative for the test allele. (Note that the size of these ellipses also represents a variation in fluorescence frequently equivalent to 5 standard deviations from the mean, while in most cases the observed differences are less than 2 standard deviations from the mean among these negative samples).

We anticipate that the typing of selected alleles or groups of alleles for DRB1, DQB1, HLA-A and HLA-B will be possible using two full 96-well trays for a single individual. If the trays are set up in advance, such an assay could be performed in less than 2 1/2 hours including DNA extraction (15 min), 2-step PCR (1 1/4 hours), fluorescence measurements (20 min/tray), and data analysis with allele designation (10 min).

References

1. Spielman R, Lee J, Bodmer W, Bodmer J, Trowsdale J. Six HLA-D region alpha chain genes of human chromosome 6: polymorphisms and associations of DC alpha related sequences with DR types. *Proc Natl Acad Sci USA* 1984:81:3461-3465.
2. Cohen D, LeGall I, Marcadet A, Font MP, Lalouel JM, Dausset J. Clusters of HLA class II beta restriction fragments describe allelic series. *Proc Natl Acad Sci USA* 1984:81:7870-7874.
3. Cascino I, Rosenshire S, Turco E, Marrari M, Duquesnoy RJ, Trucco M. Relationship between DQ alpha and DQ beta RFLP and serologically defined class II HLA antigens. *J Immunogenet* 1986:13:387-400.
4. Carsson B, Wallin J, Bohme J, Moller E. HLA-DR-DQ haplotypes defined by restriction fragment length analysis: correlation to serology. *Human Immunology* 1987:20:95-113.
5. Trucco M, Ball E. RFLP analysis of DQ-beta chain gene: Workshop report. In: *Histocompatibility Testing 1987*. Vol.1:1989:860-867
6. Mullis KB, Faloona FA. Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. In: Diego RW, ed., *Methods in Enzymology*, Academic Press, San Diego, CA, 1987:335-350.
7. Saiki RK, Gelfand OH, Stoffel S et al. Primer-directed enzymatic amplificiation of DNA with a thermostable DNA polymerase. *Science* 1989:239:487-491.
8. Trucco G, Fritsch R, Giorda R, Trucco M. Rapid detection of IDDM susceptibility, using amino acid 57 of the HLA-DQ beta chain as a marker. *Diabetes* 1989:38:1617-1622.
9. Nomura N, Ota M, Tsuji K, Inoko H. HLA-DQB1 genotyping by a modified PCR-RFLP method combined with group-specific primers. *Tissue Antigens* 1991: 38:53-59.
10. Ota M, Seki T, Nomura N, et al. Modified PCR-RFLP method for HLA-DPB1 and -DQA1 genotyping. *Tissue Antigens* 1991:38:60-71.
11. Tong JY, Hsia S, Parris GL, Nghiem DD, Cottington EM, Rudert WA, Trucco M. Molecular compatibility and renal graft survival: the HLA DQB1 genotyping. *Transplantation* 1993:55:390-395.
12. Hsia S, Tong JY, Parris GL, Nghiem DD, Cottington EM, Rudert WA, Trucco M. Molecular compatibility and renal graft survival: the HLA DRB1 genotyping. *Transplantation* 1993:55:395-399.
13. Saiki RK, Bugawan TL, Horn GT, Mullis KB, Erlich HA. Analysis of enzymatically amplified beta-globin and HLA-DQ alpha DNA with allele-specific oligonulceotide probes. *Nature* 1986:324:163-166.
14. Todd JA, Acha-Orbea H, Bell JI et al. A molecular basis for MHC class II-associated autoimmunity. Science 1988:240:1003-1009.
15. Morel PA, Dorman JS, Todd JA, McDevitt HO, Trucco M. Aspartic acid at position 57 of the HLA-DQ beta chain protects against type I diabetes: a family study. *Proc Natl Acad Sci USA* 1988:85:8111-8116.
16. Saiki RK, Walsh PS, Levenson CH, Erlich HA. Genetic analysis of amplified DNA with immobilized sequence-specific oligonulceotide probes. *Proc Natl Acad Sci USA* 1989:86:6230-6234.
17. Rudert WA, Trucco M. DNA polymers of protein binding sequences generated by PCR. *Nucl Acid Res* 1990:18:6460.
18. Rudert WA, Trucco M. Rapid detection of sequence variations using polymers of specific oligonucleotides. *Nucl Acid Res* 1992:5:1146.
19. Olerup O, Zetterquist H. HLA-DR typing by PCR amplification with sequence-specific primers (PCR-SSP) in 2 hours: an alternative to serological DR typing in clinical practice including donor-recipient matching in cadaveric transplantation. *Tissue Antigens* 1992:39:225-235.
20. Olerup O, Aldener A, Fogdell A. HLA-DQB1 and -DQA1 typing by PCR amplification with sequence-specific primers (PCR-SSP) in 2 hours. *Tissue Antigens* 1993:41:119-134.
21. Bunce M, Taylor CJ, Welsh KI. Rapid HLA-DQB typing by eight polymerase chain reaction amplifications with sequence-specific primers (PCR-SSP). *Human Immunology* 1993:37:201-206.
22. Santamaria P, Boyce-Jacino MT, Lindstrom AL, Barbosa JJ, Faras AJ, Rich SS. HLA-class II "typing": direct sequencing of DRB, DQB, and DQA genes. *Hum Immunol* 1992:33:69-81.
23. Imanishi T, Akaza T, Kimura A, Tokunaga K, Gojobori T. Allele and haplotype frequencies for HLA and complement loci in various ethnic groups. In: Tsuji K, Aizawa M, Sasazuki T (Eds.), HLA 1991: *Proceedings of the Eleventh International Histocompatibility Workshop and Conference.* Oxford: Oxford University Press, 1992:1065-1220.
24. Aizawa M. Antigens and gene frequencies of ethnic groups. In: Aizawa M, Natori T, Wakisaka A, Konoeda Y (Eds.), *HLA in Asia-Oceana.* Sapporo: Hokkaido University Press, 1986:1079-1091.
25. Browning MJ, Krausa P, Rowan A, Bicknell DC, Bodmer JG, Bodmer WF. Tissue typing the HLA-A locus from genomic DNA by sequence-specific PCR: comparison of HLA genotype and surface expression on colorectal tumor cell lines. Proc Natl Acad Sci USA 1993:90:2842-2845.
26. Krausa P, Bodmer JG, Browning MJ. Defining the common subtypes of HLA A9, A10, A28 and A19 by use of ARMS/PCR. *Tissue Antigens* 1993:42:91-99.
27. Sadler AM, Petronzelli F, Krausa P, et al. Low-resolution DNA typing for HLA-B using sequence-specific primers in allele- or group-specific ARMS/PCR. *Tissue Antigens* 1994:44:148-154.
28. Bunce M, Welsh KI: Rapid DNA typing for HLA-C using sequence-specific primers (PCR-SSP): Identification of serological and non-serologically defined HLA-C alleles including several new alleles.*Tissue Antigens* 1994:43:7-17.
29. Newton CR, Graham A, Heptinstall LE, et al. Analysis of any point mutation in DNA. The amplifcation refractory mutation system (ARMS). *Nucleic Acids Research* 1989:17:2503-2516.
30. Livak KJ, Marmaro J, Todd JA: Towards fully automated genome-wide polymorphism screening. Nature Genetics 1995:9:341-342.
31. Faas SJ, Menon R, Braun ER, Rudert WA, Trucco M. Sequence-specific priming and exonuclease-released fluorescence detection of HLA-DQB1 alleles. *Tissue Antigens* 1996:48:97-112.
32. Holland PM, Abramson RD, Waton R, Gelfand DH. Detection of specific polymerase chain reaction product by utilizing the 5' to 3' exonuclease activity of Thermus aquaticus DNA polymerase. *Proc Natl Acad Sci* 1991:88:7276-7280.

33. Maniatis T, Fritsh EF, Sambrook J. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. 1982.
34. HLA class I SSP ARMS-PCR typing kit reference manual: 12th International Histocompatibility Workshop. Distributed by the Tissue Antigen Laboratory, London, 1995.
35. Menon R, Rudert WA, Braun ER, Jaquins-Gerstl A, Faas SJ, Trucco M. Sequence-specific priming and exonuclease-released fluorescence assay for a rapid and reliable HLA-A molecular typing. *Molecular Diagnosis* 1997:2:99-111.
36. Arnett KL, Parham P. HLA class I nucleotide sequences. *Tissue Antigens* 1995:45:217-257.
37. Rudert WA, Braun ER, Faas SJ, Menon R, Jaquins-Gerstl A, Trucco M. Double-labeled fluorescent probes for 5′ nuclease assays: purification and performance evaluation. *BioTechniques* 1997:22:1140-1145.
38. Anai M, Hirahashi T, Takagi Y. A deoxyribonuclease which requires nucleoside triphosphate from Micrococcus lysodeikticus. I. Purification and characterization of the deoxyribonuclease activity. *J Biol Chem* 1970:245:767-774.
39. XIth International HLA Workshop DNA Component. Reference protocols (general remarks) Fukuoka, Japan, July 1990.
40. Livak KJ, Flood SJA, Marmaro J, Giusti W, Deetz K. Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for detecting PCR product and nucleic acid hybridization. *PCR Methods & Applications* 1995:4:1-6.
41. Lee LG, Connell CR, Bloch W. Allelic discrimination by nick-translation PCR with fluorogenic probes. *Nucl Acids Research 1993:21:3761-3766.*
42. Smith S, Taylor CJ. Discrepant sequence at codon 57 of DQB1: implication on HLA typing of "Asp 57" in IDDM. *Tissue Antigens* 1995:46:71-72.
43. Bodmer JG, Marsh SGE, Albert ED et al. Nomenclature for factors of the HLA system, 1995. *Tissue Antigens* 1995:46:1-18.

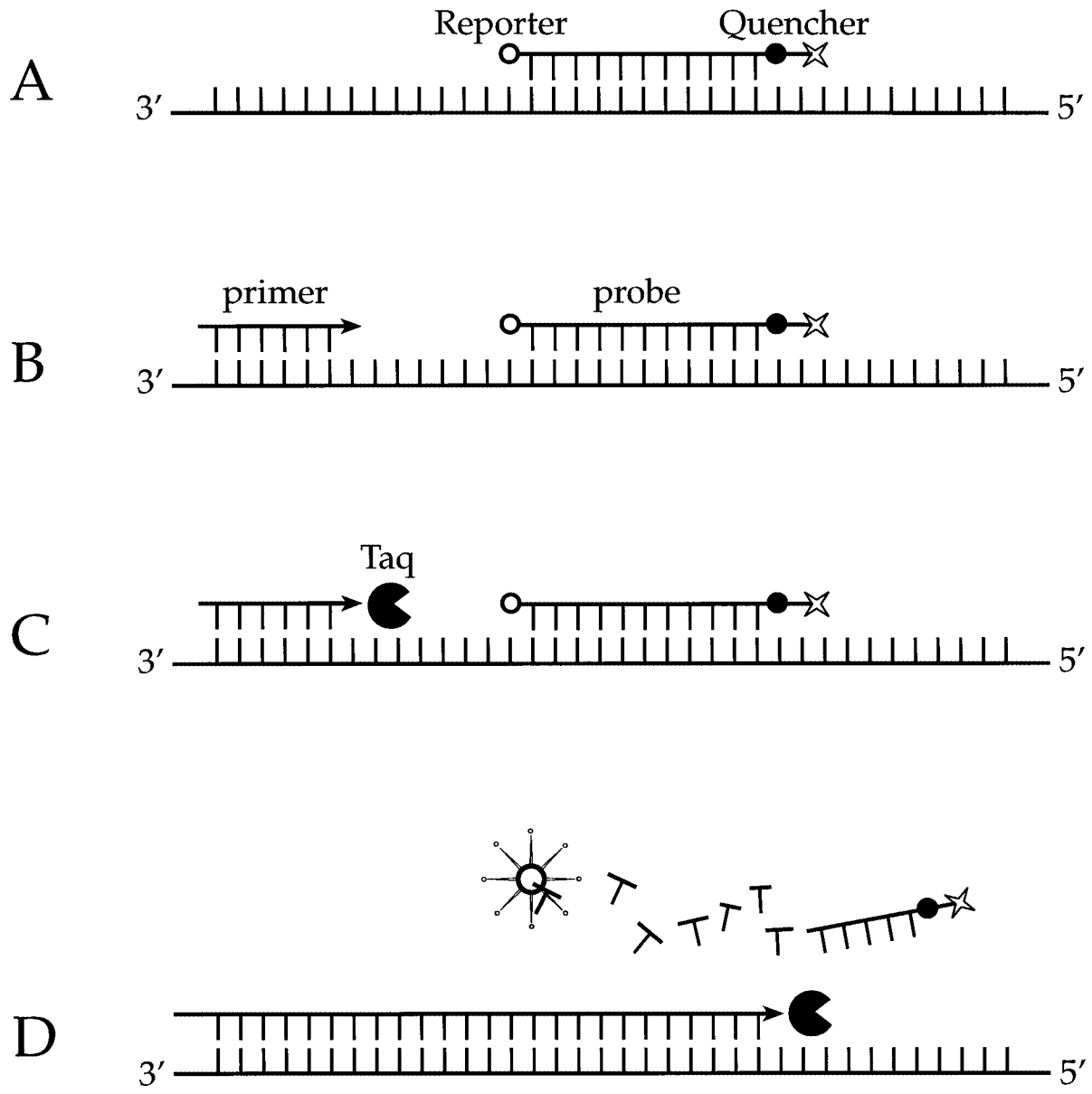


Figure 1. Schematic of sequence-specific priming and exonuclease-released fluorescence detection. A) Hybridization of a doubly-labeled fluorogenic probe to target DNA sequences during PCR amplification. The positions of the reporter dye (FAM or HEX) and the quencher dye (TAMRA) are indicated by the symbols ○ and ●, respectively. The 3′ phosphate is indicated by the ◈. The increased length of the probes (relative to that of the primers) favors the hybridization of the probe prior to the annealing of the primers. B) Primer annealing to the template DNA. C) Extension of the primer by Taq1 polymerase. D) Encounter of the double-stranded template formed by hybridization of the probe initiates cleavage of the probe at its 5′ end by Taq 1 polymerase. Cleavage of the probe physically separates the reporter and quencher dye molecules, which abrogates the quencher effect and results in an increase in reporter fluorescence. (From ref. 31)

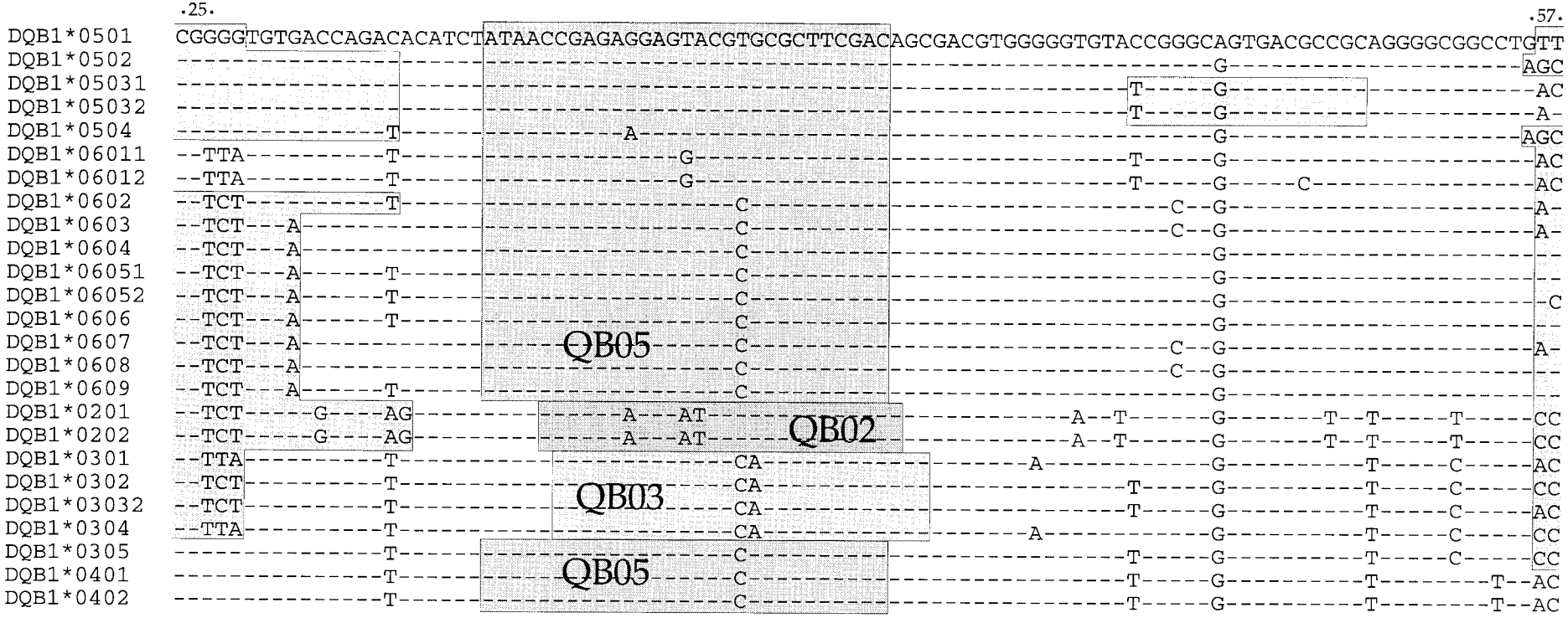


Figure 2. Location of the DQB1 probes, QB02, QB03, and QB05, used for the SSPERF method. The area shown lies between codons 25 and 57 of the DQB1 gene (indicated). The primers used for allele-specific amplification have been described in detail in Table V and those that overlap with this portion of the DQB1 gene are indicated by shaded areas 5′ and 3′ of the indicated probes. (From ref. 31)

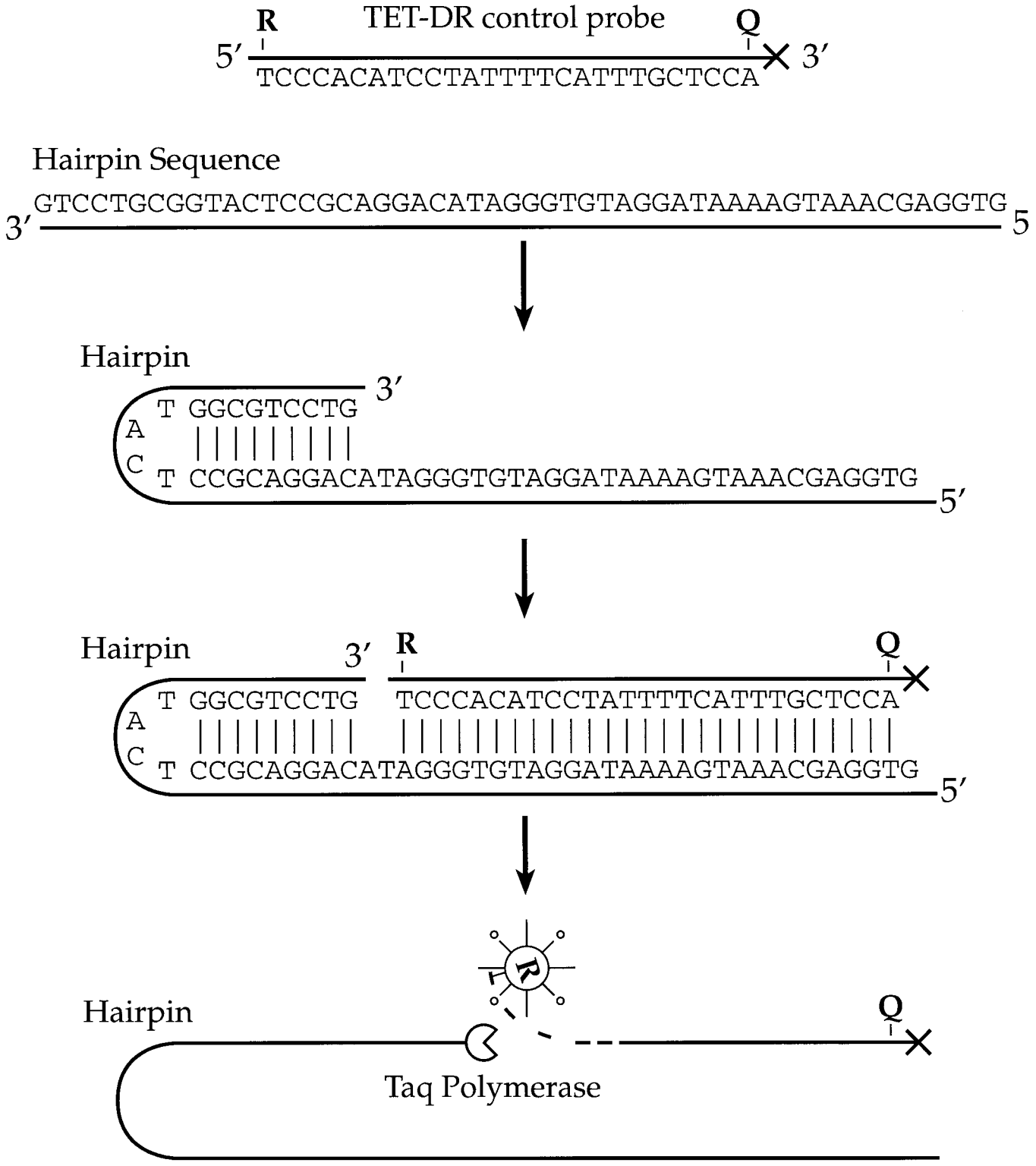


Figure 3. Schematic of the hairpin assay to evaluate fluorogenic probe performance. The sequence of the TET-labeled fluorogenic probe used as a control on DRB typing, illustrates the design of its complementary hair pin primer. The primer forms a spontaneous hairpin at the 3′ end and hybridizes to its complementary sequence within the fluorescent probe. During primer extension, Taq DNA polymerase cleaves the 5′ end of the probe, liberating the reporter dye from the quenching effect of the 3′ dye. (From ref. 37)

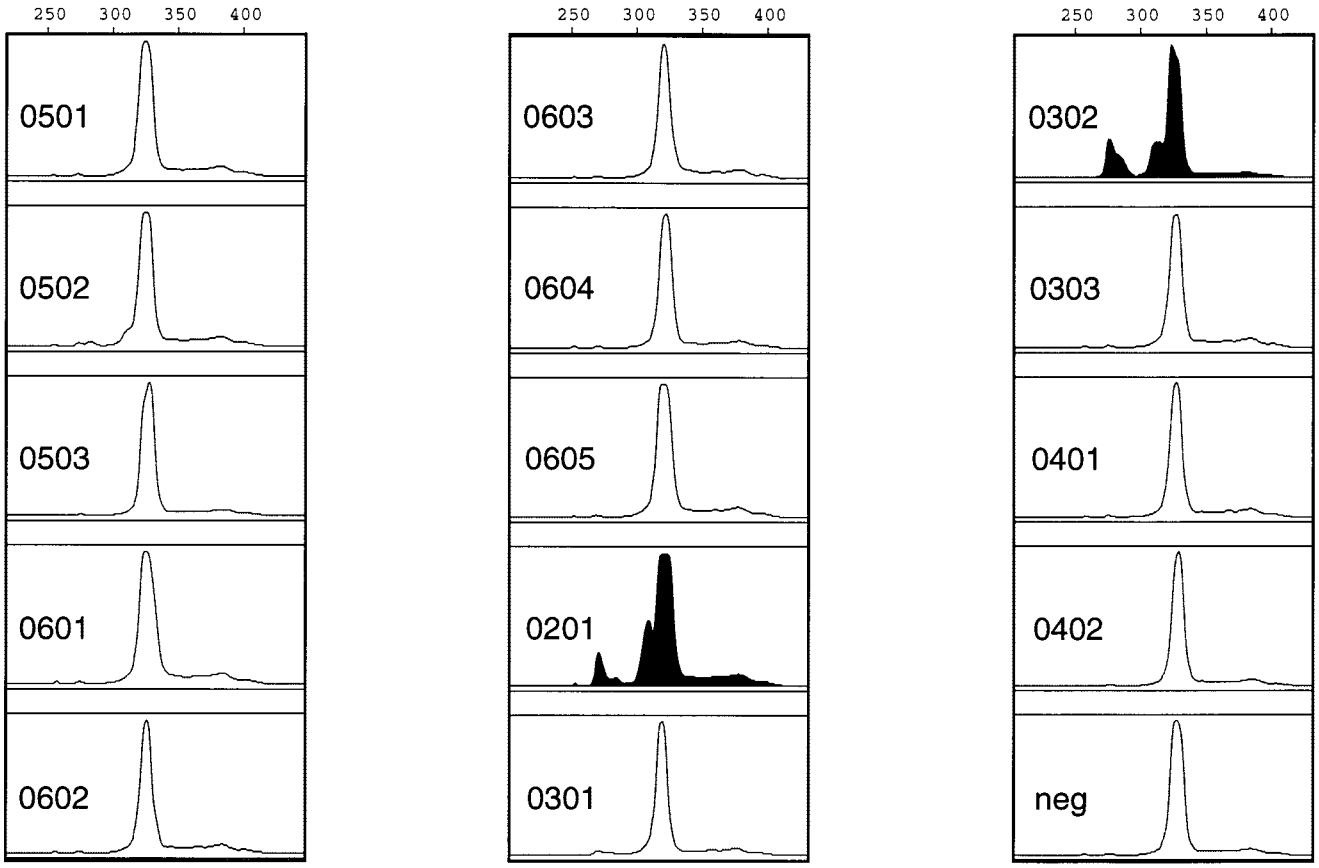


Figure 4. Specific cleavage of the appropriate fluorogenic probe under conditions of SSP-PCR amplification of DNA from a heterozygous (DQB1*0201/*0302) sample. PCR reactions were performed using the allele-specific primer pairs and probe combinations described in Table V. The negative control consisted of all of the elements of the PCR reaction except template DNA. The scale at the top of the panel indicates the relative position of the peaks in the gel expressed as scan numbers. Reactions exhibiting probe cleavage are highlighted in black. The intact probe migrates as a single peak detected at approximately scan #325, while cleavage products migrate faster in the gel and appear as smaller peaks between scan #260 and #320. The high ratio of intact to cleaved probe reflects the fact that the probe is added to each PCR reaction in large excess. (From ref. 31)

Experimental Design

	1	2	3	4	5	6	7	8	9	10	11	12
A	PCR	~~QB05~~ DRCON	QBO5 ~~DRCON~~	~~QB05~~ ~~DRCON~~	QBO2 DRCON	QBO5 DRCON	QBO2 DRCON	QB03 DRCON	QB03 DRCON	QB03 DRCON	QBO5 DRCON	QBO5 DRCON
B	QBO5 DRCON 5.01,3.01	QBO5 DRCON 5.01,3.01	QBO5 DRCON 5.01,3.01	QBO5 DRCON 5.01,3.01	QBO5 DRCON 5.01,3.01	QBO5 DRCON 5.01,3.01	QBO5 DRCON 5.13,3.06	QBO5 DRCON 5.13,3.06	QBO5 DRCON 5.13,3.06	QBO5 DRCON 5.13,3.06	QBO5 DRCON 5.13,3.06	QBO5 DRCON 5.13,3.06
C	QBO5 DRCON 5.02,3.02	QBO5 DRCON 5.02,3.02	QBO5 DRCON 5.02,3.02	QBO5 DRCON 5.02,3.02	QBO5 DRCON 5.02,3.02	QBO5 DRCON 5.02,3.02	QBO2 DRCON 5.07,3.07	QBO2 DRCON 5.07,3.07	QBO2 DRCON 5.07,3.07	QBO2 DRCON 5.07,3.07	QBO2 DRCON 5.07,3.07	QBO2 DRCON 5.07,3.07
D	QBO5 DRCON 5.02,3.03	QBO5 DRCON 5.02,3.03	QBO5 DRCON 5.02,3.03	QBO5 DRCON 5.02,3.03	QBO5 DRCON 5.02,3.03	QBO5 DRCON 5.02,3.03	QBO3 DRCON 5.09,3.09	QBO3 DRCON 5.09,3.09	QBO3 DRCON 5.09,3.09	QBO3 DRCON 5.09,3.09	QBO3 DRCON 5.09,3.09	QBO3 DRCON 5.09,3.09
E	QBO5 DRCON 5.03,3.04	QBO5 DRCON 5.03,3.04	QBO5 DRCON 5.03,3.04	QBO5 DRCON 5.03,3.04	QBO5 DRCON 5.03,3.04	QBO5 DRCON 5.03,3.04	QBO3 DRCON 5.08,3.08	QBO3 DRCON 5.08,3.08	QBO3 DRCON 5.08,3.08	QBO3 DRCON 5.08,3.08	QBO3 DRCON 5.08,3.08	QBO3 DRCON 5.08,3.08
F	QBO5 DRCON 5.04,3.05	QBO5 DRCON 5.04,3.05	QBO5 DRCON 5.04,3.05	QBO5 DRCON 5.04,3.05	QBO5 DRCON 5.04,3.05	QBO5 DRCON 5.04,3.05	QBO3 DRCON 5.08,3.10	QBO3 DRCON 5.08,3.10	QBO3 DRCON 5.08,3.10	QBO3 DRCON 5.08,3.10	QBO3 DRCON 5.08,3.10	QBO3 DRCON 5.08,3.10
G	QBO5 DRCON 5.05,3.05	QBO5 DRCON 5.05,3.05	QBO5 DRCON 5.05,3.05	QBO5 DRCON 5.05,3.05	QBO5 DRCON 5.05,3.05	QBO5 DRCON 5.05,3.05	QBO5 DRCON 5.10,3.11	QBO5 DRCON 5.10,3.11	QBO5 DRCON 5.10,3.11	QBO5 DRCON 5.10,3.11	QBO5 DRCON 5.10,3.11	QBO5 DRCON 5.10,3.11
H	QBO5 DRCON 5.06,3.06	QBO5 DRCON 5.06,3.06	QBO5 DRCON 5.06,3.06	QBO5 DRCON 5.06,3.06	QBO5 DRCON 5.06,3.06	QBO5 DRCON 5.06,3.06	QBO5 DRCON 5.11,3.11	QBO5 DRCON 5.11,3.11	QBO5 DRCON 5.11,3.11	QBO5 DRCON 5.11,3.11	QBO5 DRCON 5.11,3.11	QBO5 DRCON 5.11,3.11
p:	1	2	3	4	5	6	1	2	3	4	5	6

FAM

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.086	335.443	59.513	343.912	28.915	40.681	32.562	37.637	86.009	26.563	32.231	31.888
B	72.882	248.266	79.573	297.952	80.964	82.198	69.151	68.510	64.126	46.699	57.824	66.677
C	69.948	68.503	73.595	78.290	79.028	79.086	42.544	47.964	47.027	169.938	43.837	204.938
D	71.047	72.601	75.268	77.989	78.420	78.001	57.393	65.390	50.436	60.848	64.018	63.130
E	79.592	77.426	79.496	85.706	84.346	84.484	89.333	97.923	347.519	92.204	327.134	87.711
F	115.771	78.758	78.591	81.537	82.790	82.682	202.683	53.055	51.826	56.942	54.991	51.504
G	85.046	83.323	84.531	85.061	302.259	337.683	81.629	89.281	84.558	89.006	82.657	86.816
H	87.855	90.420	87.383	92.537	87.922	89.869	200.142	236.200	84.960	93.976	89.677	82.533
p:	1	2	3	4	5	6	1	2	3	4	5	6

HEX

	1	2	3	4	5	6	7	8	9	10	11	12
A	-0.071	112.750	96.767	172.637	28.226	33.216	31.319	31.766	37.001	25.712	28.658	28.067
B	54.747	92.927	59.298	103.201	59.580	57.681	59.927	58.406	56.116	40.210	50.780	57.568
C	62.168	56.484	64.947	67.900	68.146	66.373	50.288	56.714	57.943	80.730	54.643	82.955
D	65.427	63.379	69.904	70.636	71.335	69.065	55.560	62.486	48.849	59.329	61.827	60.378
E	70.213	67.633	68.081	74.510	73.292	72.587	64.222	68.827	111.474	67.287	108.579	65.261
F	77.201	68.934	69.513	71.646	73.994	71.801	86.479	60.162	60.698	61.864	61.593	57.743
G	72.288	70.657	71.807	70.052	119.480	125.060	70.714	79.352	75.964	78.654	72.411	73.862
H	80.911	80.010	77.627	82.591	77.545	76.053	95.973	115.605	76.963	87.829	81.769	70.993
p:	1	2	3	4	5	6	1	2	3	4	5	6

TAMRA

	1	2	3	4	5	6	7	8	9	10	11	12
A	-0.030	65.016	71.565	80.605	32.929	43.975	35.649	35.825	34.072	31.292	38.815	38.562
B	55.410	56.087	58.357	54.225	59.274	57.916	60.379	59.754	55.737	40.307	52.287	60.941
C	63.465	58.428	65.387	68.783	69.723	69.387	43.200	49.763	50.239	54.254	48.562	53.124
D	65.507	64.253	68.602	70.069	72.023	70.177	47.200	52.680	40.152	50.016	52.612	52.123
E	71.049	68.352	68.034	74.940	74.881	75.659	51.213	54.457	56.236	53.356	57.379	53.655
F	70.117	70.198	69.866	71.905	75.244	74.520	55.682	53.096	52.240	54.463	53.828	52.140
G	72.474	72.146	71.759	70.503	74.757	73.175	74.067	80.781	75.925	77.963	73.516	76.509
H	79.370	79.348	76.538	81.831	78.523	77.525	71.364	85.883	75.018	84.217	80.547	72.752
p:	1	2	3	4	5	6	1	2	3	4	5	6

Figure 5. A) Experimental layout of a typical assay in which 6 sample DNAs are analyzed for DQB1 in a single 96-well tray. Row A contains various probe controls as indicated. DRCON refers to the DR intron control probe. QB02, QB03 and QB05 refer to the DQB1 specific probes. Wells A2-A4 contain mixtures of equal amounts of intact or ATP-dependent DNAse-digested (e.g.,QB05) probes at a final concentration of 50nM. Wells A5-A12 lack template DNA but contain allele-specific and control primers and the two indicated fluorogenic probes in 1x PCR buffer. (Note that the unusual order of these control probes is simply to enable the multichannel pipetteting of a group of horizontal probe master mixes that correspond, in order, to the 7 vertical master mixes containing the same probes). The primer pairs used to amplify each allele are indicated. The patient number is indicated at the bottom of each column (e.g DNA from Patient #1 is distributed in Column 1, wells B-H and in Column 7, wells B-H). Raw fluorescence values for FAM (B), HEX (C) and TAMRA (D) are direct readings from the LS-50B fluorescence spectrometer prior to normalization. The shaded wells highlight an increase in FAM fluorescence that indicates the amplification and specific detection of the test allele. Note the minor increase in the FAM fluorescence value associated with non-specific amplification of the *0303 allele by the B-5'04/B-3'05 (e.g., the *0602-specific) primer pair (indicated in well F1, by hatched lines). (From ref. 31)

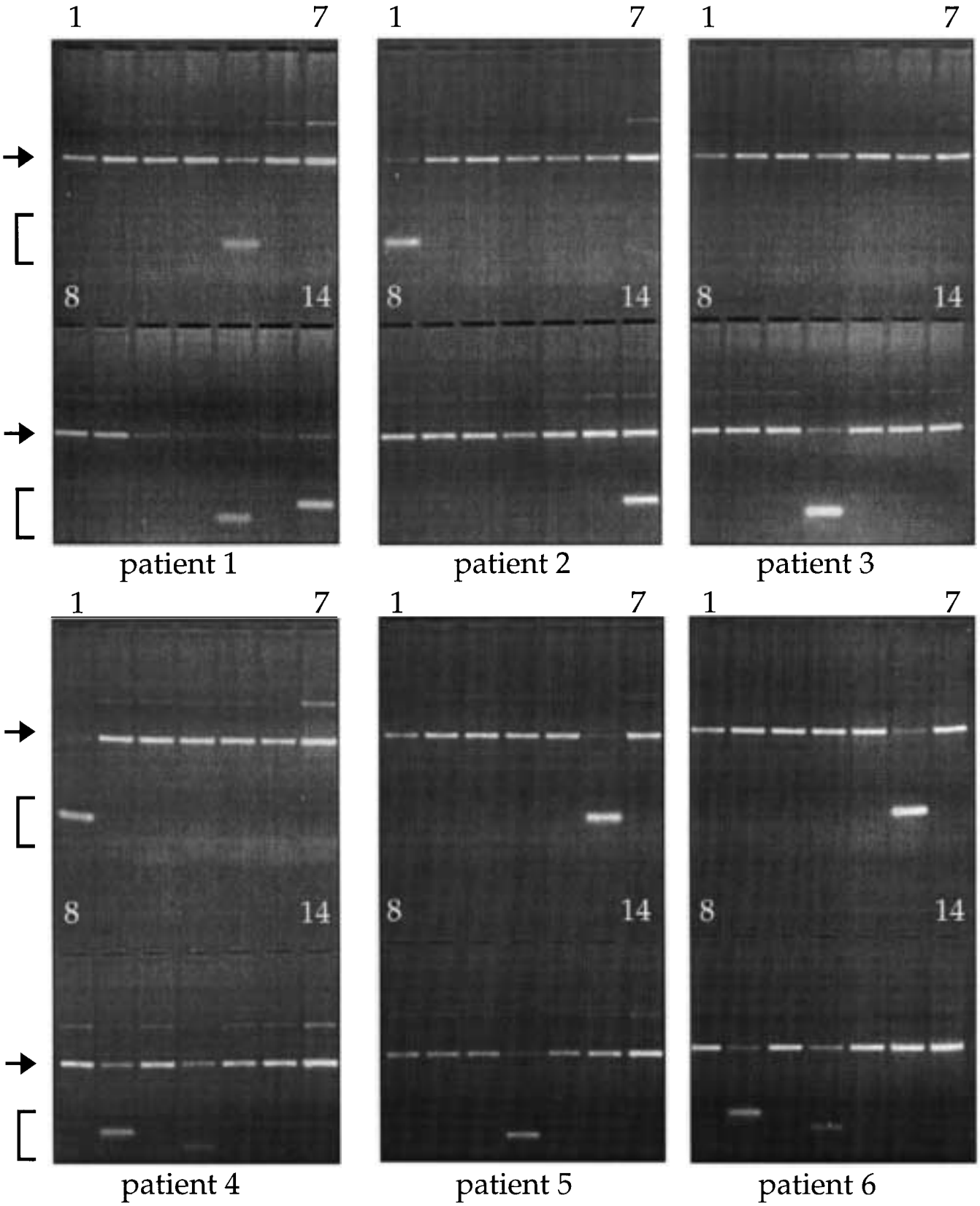


Figure 6. Agarose gel electrophoresis of the samples analyzed in Fig. 5. An aliquot of the amplified products from each PCR reaction was resolved by gel electrophoresis. For each patient, amplifications generated by primer pairs 1-7 (listed in Table V and Fig.5) are shown in the top seven wells and those generated by primer pairs 8-14 are shown in the bottom seven wells. Bands in each well representing amplifications of the DRB1 intron internal control are indicated by the arrow. Bands representing allele-specific amplifications are included in the area in each gel indicated by the bracket. Weak cross-reactive bands are evident in samples from Patient #1 (well 5), Patient #4 (well 11) and Patient #6 (well 11) and are discussed in the text. (From ref. 31)

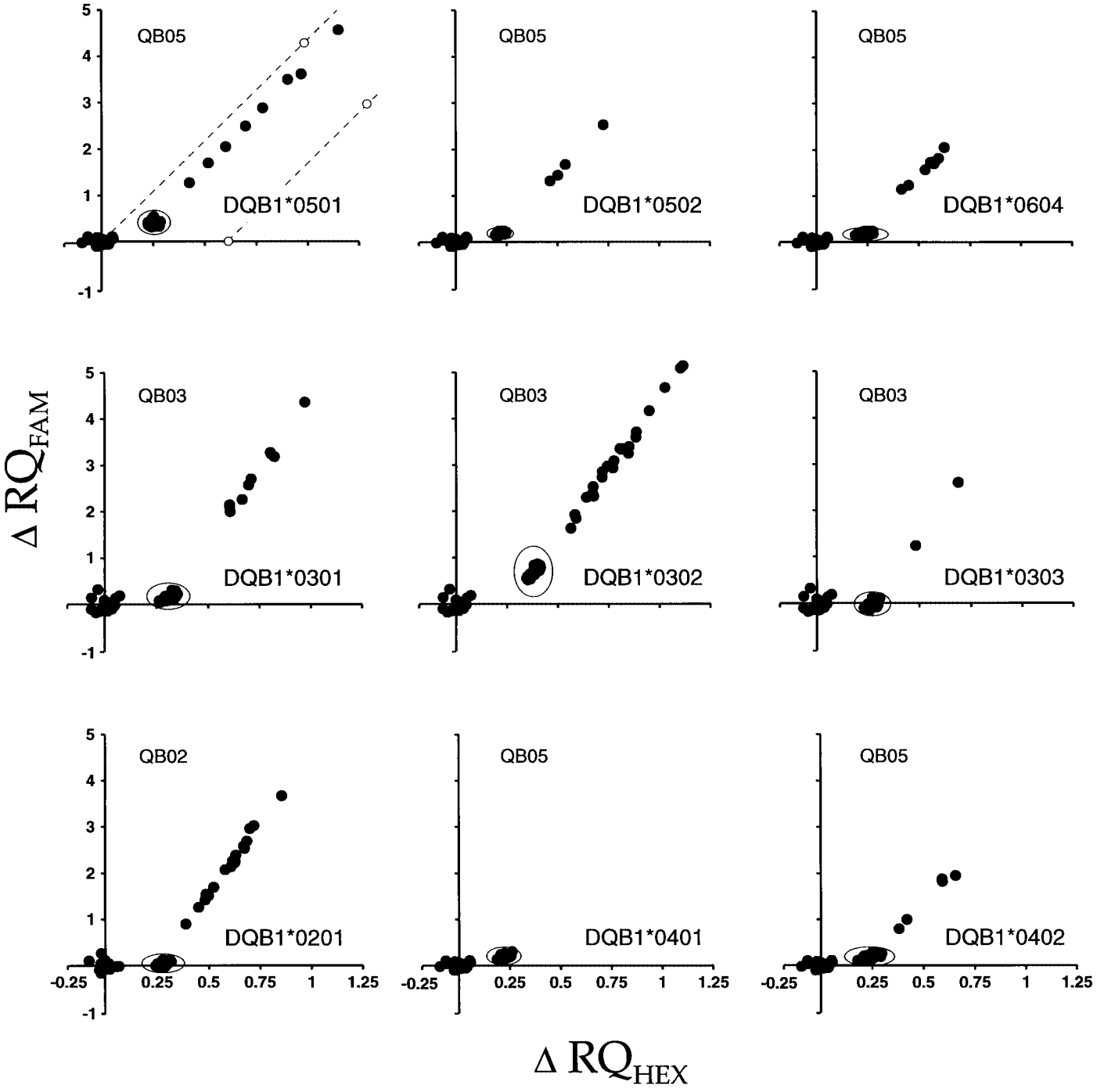


Figure 7. Fluorescence data for several DQB1 alleles from 50 samples. In each graph all 50 sample are represented. Each data point represents the ΔRQ_{FAM} and ΔRQ_{HEX} normalized fluorescence values for a single patient. The scales on both abscissa and ordinate are identical for all graphs. The test allele as well as the DQB1-specific probe used for its detection are indicated. Normalized baseline fluorescence of the intact probes (i.e., probes alone) are represented by the cluster of points at the origin of each graph. Fluorescence associated with the negative amplification of the indicated test allele in the presence of amplification of the DRB1 intron internal control from the 50 samples is represented by the encircled cluster of points. The size of the ellipse around these control amplifications represents 5 standard deviations from the mean fluorescence of these reactions. Increased fluorescence, associated with amplifications specific for the indicated test allele, is represented by individual data points in a diagonal orientation (for example, 8 patients were positive for the DQB1*0501 allele, 4 were positive for the DQB1*0502, etc.). These points fall within a range (e.g., delineated by the dotted lines flanking the data for test allele *0501), corresponding to the values (empty circles) of the combinations of intact and digested probes (e.g., wells A2, A3, and A4 of experimental lay-out in Fig.5). (From ref. 31)

Table I. HLA-A Primers and Probes for TaqMan Method

PROBE: HLA-AI *5' FAM-CCCTgCgCggCTACTACAACCAgAgCgAgg-L(TAMARA)$PO_4$3'*

SPECIFICITY	5' PRIMER	5' SENSE 3'	3' PRIMER	5' ANTISENSE 3'
0101/02	12WSAL#16	ggACCAggAgACACggAATA	12WS/AL#Z	AggTATCTgCggAgCCCg
3601	12WS/A15	CgACgCCgCgAgCCAgAA	12WS/A-3601	gAgCCACTCCACgCACgT
03	12WS/AL#7	gCgACgCCgCgAgCCA	12WS/AL#D	CACTCCACgCACgTgCCA
25,2605	12WS/AL#11	CACAgACTgACCgAgAgAg	12WS/AL#C'	ggATgTAATCCTTgCigTCgTAA
2601/02/04/07/08,4301	12WS/AL#34	ACTCACAgACTgACCgAg	12WS/AL#C'	ggATgTAATCCTTgCigTCgTAA
25,26,34,66	12WS/AL#4	gAgTATTgggACCggAAC	12WS/AL#C'	ggATgTAATCCTTgCigTCgTAA
2502,34,6601/02	12WS/AL#6	CggAATgTgAAggCCCAg	12WS/AL#C'	ggATgTAATCCTTgCigTCgTAA
4301	12WS/AL#17	CCggAgTATTgggACCTgC	12WS/AL#C'	ggATgTAATCCTTgCigTCgTAA
1101/02/04,2608	12WS/AL#6	CggAATgTgAAggCCCAg	12WS/AL#I	CTCTCTgCTgCTCCgCCg
68011/012/02,6901	12WS/AL#6	CggAATgTgAAggCCCAg	12WS/AL#H	CCAAgAgCgCAggTCCTCT
1101-04,68,69	12WS/AL#6	CggAATgTgAAggCCCAg	145a	TgCTTggTggTCTgAgCT
3402,6801/03-05	12WS/AL#4	gAgTATTgggACCggAAC	12WS/AL#AM	CgTCgTAggCgTCCTgCC
6802	12WS/AL#25	TgAggTATTTCTTCACCTCCA	12WS/AL#H	CCAAgAgCgCAggTCCTCT
6901	12WS/AL#6	CggAATgTgAAggCCCAg	12WS/AL#Y	CCgCggAggAAgCgCCA
29	12WS/AL#35	AggATggAgCCgCgggCA	12WSAL#F'	gCgCAggTCCTCiTTCAA
30	12WS/AL#12	CCggCCCggCAgTggA	12WS/AL#G	CCgTCgTAggCgTgCTgT
33	12WS/AL#4	gAgTATTgggACCggAAC	12WS/AL#F'	gCgCAggTCCTCiTTCAA
32,74	12WS/AL#24	CACgCAgTTCgTgCggTTT	12WS/AL#AR	CTggTACCCgCggAggAg
29(?),31,33,74	12WS/AL#32	CCgAgTggACCTggggAC	12WS/AL#F"	gCgCAggTCCTCiTTCAA
8001	12WS/AL#54	gAAggCCCACTCACAgACTA	12WS/AL#BK	gAgCCCgTCCACgCACTC
02,23,24,68,69	12WS/AL#30	CggAATgTgAAggCCCACT	12WS/AL#H	CCAAgAgCgCAggTCCTCT
	12WS/AL#31	ACggAATgTgAAggCCCAgT		
all but 02,23,24,68,69	12WS/AL#30	CggAATgTgAAggCCCACT	12WS/AL#L	CAAgAgCgCAggTCCTCg
	12WS/AL#31	ACggAATgTgAAggCCCAgT		

PROBE: HLA-AII *5' FAM-CgCTTCATCgCAgTgggCTACgTggACgAC-L(TAMARA)$PO_4$3'*

SPECIFICITY	5' PRIMER	5' SENSE 3'	3' PRIMER	5' ANTISENSE 3'
02	12WS/AL#37	TCCTCgTCCCCAggCTCT	12WS/AL#AW	gTggCCCCTggTACCCgT

PROBE: HLA-AIII *5' FAM-CgCTCCgCTACTACAACCAgAgC-L(TAMARA)$PO_4$3'*

SPECIFICITY	5' PRIMER	5' SENSE 3'	3' PRIMER	5' ANTISENSE 3'
2301,2413	12WS/AL#8	gCCggAgTATTgggACgA	12WS/AL#Q	CCTCCAggTAggCTCTCAA
2402-05/07/09/10/14	12WS/AL#8	gCCggAgTATTgggACgA	12WS/AL#R	CCTCCAggTAggCTCTCTg
all 24's except 2405	12WS/AL#11	CACAgACTgACCgAgAgAg	159a	CgCCTCCCACTTgCgCTT
25,2605	12WS/AL#11	CACAgACTgACCgAgAgAg	12WS/AL#C'	ggATgTAATCCTTgCigTCgTAA
25,26,34,66	12WS/AL#4	gAgTATTgggACCggAAC	12WS/AL#C'	ggATgTAATCCTTgCigTCgTAA
2502,34,6601/02	12WS/AL#6	CggAATgTgAAggCCCAg	12WS/AL#C'	ggATgTAATCCTTgCigTCgTAA
31	12WS/AL#10	gATAgAgCAggAgAggCCT	12WS/AL#F'	gCgCAggTCCTCiTTCAA
32	12WS/AL#11	CACAgACTgACCgAgAgAg	12WS/AL#F'	gCgCAggTCCTCiTTCAA
32,74	12WS/AL#24	CACgCAgTTCgTgCggTTT	12WS/AL#AR	CTggTACCCgCggAggAg
02,23,24,68,69	12WS/AL#30	CggAATgTgAAggCCCACT	12WS/AL#H	CCAAgAgCgCAggTCCTCT
	12WS/AL#31	ACggAATgTgAAggCCCAgT		
all but 02,23,24,68,69	12WS/AL#30	CggAATgTgAAggCCCACT	12WS/AL#L	CAAgAgCgCAggTCCTCg
	12WS/AL#31	ACggAATgTgAAggCCCAgT		

Table II. HLA-B Primers and Probes for TaqMan Method

PROBE: HLA-BI *5' FAM-AACCTgCgCggCTACTACAACCAgAgCgAgg-L(TAMARA)$PO_4$3'*

SPECIFICITY	5'PRIMER	5' SENSE 3'	3' PRIMER	5' ANTISENSE 3'
0702-08,8101	12ws/B#5c	ggAgTATTgggACCggAAC	12ws/G1c	TACCAgCgCgCTCCAgCT
0702-08,40011-12/07,4801/03	24g*	CCTCCTgCTgCTCTCggC	12ws/G1c	TACCAgCgCgCTCCAgCT
0801-04	12ws/B#5g	GACCggAACACACAgATCTT	12ws/B#g1b	CCgCgCgCTCCAgCgTg
1301-03	12ws/B#4d	CgCgAgTCCgAggATggC	12ws/b#c3	ATCCTTgCCgTCgTAggCT
1401/02	12ws/B#B14	gAgCAggAggggCCggAA	12ws/AL B#I	CgTCgCAgCCATACATCCA
1501/03-07/12/14/19/20/ 24-27/32-35,4003,4808	12ws/B#5b	ACCgggAgACACAgATCTC	12ws/B#c2	CCTTgCCgTCgTAggCgg
501/02/04-08/11/13/15-17/20/ 21/24-28/30/32-35,4601,5701-04	12ws/B#4d	CgCgAgTCCgAggATggC	12ws/B#F2	gAgCCACTCCACgCACAg
1509/10/18/21/23/37	12ws/5a.1	gACCggAACACACAgATCTg	12ws/B#F2	gAgCCACTCCACgCACAg
1503/18/23/29,4802,3907,72v	B209	CgCCgCgAgTCCgAgAgA	B214	CTTgCCgTCgTAggCgg
3701,4406,5108	12ws/B#4c	gCCgCgAgTCCgAggAC	12ws/AS#A	CCTCCAggTAggCTCTgTC
3801/02	B208 B196	ACCgAgAgAACCTgCggAT TACCgAgAgAACCTgCgCA	B217	CgTgCCCTCCAggTAggT
3801/02,39	271-288	TCAAgACCAACACACAgA	B217	CgTgCCCTCCAggTAggT
3901-12,37011/012	B207	CCgAgAgAgCCTgCggAA	B217	CgTgCCCTCCAggTAggT
40011-09,4701-02	12ws/B#4a.2	CgCCACgAgTCCgAggAA	12ws/B#D3	gAgCCgCCgTgTCCgCg
4011/02/07	B280	gCTACgTggACgACACgCT	B229	CTCCAACTTgCgCTgggA
4101/02	12ws/B#1a	CCACTCCATgAggTATTTCC	12ws/B#G1b	CCgCgCgCTCCAgCgTg
4402-05/07-10	12ws/B#6a	TACCgAgAgAACCTgCgC	12ws/B#F3	CCAggTATCTgCggAgCg
1514,4501,8201	B207	CCgAgAgAgCCTgCggAA	12ws/B#F3	CCAggTATCTgCggAgCg
4601	B240	gAgACACAgAAgTACAAgCg	B241	gCCgCggTCCAggAgCT
3702,4701/02	B192	ACCgggAgACACAgATCTC	B228	TCgTAggCgTCCTggTgg
40011/012,4801/03	12ws/B#5b	ACCgggAgACACAgATCTC	12ws/B#G1c	TACCAgCgCgCTCCAgCT
4802	B271	gggAgCCCCgCTTCATCT	12ws/B#K	gCCATACATCCTCTggATgA
2704/06/10,4005,4901,5001	12ws/B#1a	CCACTCCATgAggTATTTCC	B225	CTCTCAgCTgCTCCgCCT
52011/02	B192	ACCgggAgACACAgATCTC	B216	CgTTCAgggCgATgTAATCT
3501/03-09/11/14/15/17-21, 5301-02	12ws/B#5g	gACCggAACACACAgATCTT	12ws/B#A3	ggAggAggCgCCCgTCg
5401	12ws/B#54	gCgggCgCCgTgggTg	12ws/B#D1	gCCgCggTCCAggAgCT
5401,5501-03/05,5601/02,8201	B203	CAgATCTACAAggCCCAgg	12ws/B#c3	ATCCTTgCCgTCgTAggCT
5501-03/05,5601,4501	B209	CgCCgCgAgTCCgAgAgA	B236	CCATACATCgTCTgCCAA
4901,5901	B208	ACCgAgAgAACCTgCggAT	12ws/B#c3	ATCCTTgCCgTCgTAggCT
5401,5501-03/05,5601/02, 5901,8201	12ws/B#4b	ACgCCgCgAgTCCgAgAg	12ws/B#c3	ATCCTTgCCgTCgTAggCT
67011/012	B203	CAgATCTACAAggCCCAgg	B217	CgTgCCCTCCAggTAggT
7301	B30	TACTACAACCAgAgCgAggA	B236	CCATACATCgTCTgCCAA
1509,78011-022	B207	CCgAgAgAgCCTgCggAA	B216	CgTTCAgggCgATgTAATCT
8101	B203	CAgATCTACAAggCCCAgg	8101	TCCAACTTgCgCTgggA

PROBE: HLA-BII *5' FAM-CgCTCCgCTACTACAACCAgAgC-L(TAMARA)$PO_4$3'*

SPECIFICITY	5'PRIMER	5' SENSE 3'	3' PRIMER	5' ANTISENSE 3'
0801-04	12ws/B#5g	gACCggAACACACAgATCTT	12ws/B#G1b	CCgCgCgCTCCAgCgTg
1301-02	12ws/B#6a	TACCgAgAgAACCTgCgC	12ws/B#H	gggCCgCCTCCCACTTgA
1501/09-07/12/14/19/20/ 24-27/32-35,4003,4808	12ws/B#5b	ACCgggAgACACAgATCTC	12ws/B#C2	CCTTgCCgTCgTAggCgg
1501/02/04-08/11/13/15/17/20/ 21/24-28/30/32-35,4601,5701-04	12ws/B#4d	CgCgAgTCCgAggATggc	12ws/B#F2	gAgCCACTCCACgCACAg
1509/10/18/21/23/37	12ws/B#5a.1	gACCggAACACACAgATCTg	12ws/B#F2	gAgCCACTCCACgCACAg
1801-03	B187	gCgCCgTggATAgAgAggCAA	B214	CTTgCCgTCgTAggCgg

Table II. HLA-B Primers and Probes for TaqMan Method (continued)

1522,1801-03,3501-18/20-21, 7801-22	B188	gCCgCgAgTCCgAggAC	B237	GCgCAggTTCCgCAggC
3701,4406,5108	12ws/B#4c	gCCgCgAgTCCgAggAC	12ws/AS#A	CCTCCAggTAggCTCTgTC
3701,3902/08	B192	ACCgggAgACACAgATCTC	B392	CCTTgCCgTCgTAggCgA
3801/02	B208	ACCgAgAgAACCTgCggAT	B217	CgTgCCCTCCAggTAggT
	B196	TACCgAgAgAACCTgCgCA		
3901-12,67011/012	B207	CCgAgAgAgCCTgCggAA	B217	CgTgCCCTCCAggTAggT
4201	12ws/B#5A.1	gACgACACCCAgTTCgTgA	12ws/B#G1b	CCgCgCgCTCCAgCgTg
4601	B240	gAgACACAgAAgTACAAgCg	B241	gCCgCggTCCAggAgCT
4802	B271	gggAgCCCCgCTTCATCT	12ws/B#K	gCCATACATCCTCTggATgA
2704/06/10,4005,4901,5001	12ws/B#1a	CCACTCCATgAggTATTTCC	B225	CTCTCAgCTgCTCCgCCT
4002/06/08-09,4101/02, 4402-04/07/10,4901,5001	12ws/B#4a.2	CgCCACgAgTCCgAggAA	B276	TCCCACTTgCgCTgggT
1509,51011-08,7801/02	12ws/B#5c	ggAgTATTgggACCggAAC	B216	CgTTCAgggCgATgTAATCT
52011/02	B192	ACCgggAgACACAgATCTC	B216	CgTTCAgggCgATgTAATCT
5601-03,4604(?)	B203	CAgATCTCAAAggCCCAgg	12ws/B#F2	gAgCCACTCCACgCACAg
5701-04	12ws/B#G183	ggCCggAgTATTgggACg	12ws/B#K2	CgTCgCAgCCATACATCAC
5801/02	B194	AACATgAAggCCTCCgCg	B213	gAggAggCgCCCgTCg
4901,5901	B208	ACCgAgAgAACCTgCggAT	12ws/B#c3	ATCCTTgCCgTCgTAggCT

PROBE: HLA-BIII *5' FAM-CTCggACTCiTggCgTCgCTgTCgAA-L(TAMARA)PO_4 3'*

SPECIFICITY	**5'PRIMER**	**5' SENSE 3'**	**3' PRIMER**	**5' ANTISENSE 3'**
8201	B203	CAgATCTACAAggCCCAgg	8201	gCgACTCCACgCACAggT
Bw4	12ws/B#29	gACgACACgCAgTTCgTgA	12ws/B#32	CgCTCTggTTgTAgTAgCg
	12ws/B#30	gACgACACgCTgTTCgTgA		
	12ws/B#5A'	gACgACACCCAgTTCgTgA		
	12ws/B#18	gACggCACCCAgTTCgTgA		
Bw6	12ws/B#29	gACgACACgCAgTTCgTgA	12ws/B#31	CgCTCTggTTgTAg^AgCC
	12ws/B#30	gACgACACgCTgTTCgTgA		
	12ws/B#5A'	gACgACACCCAgTTCgTgA		
	12ws/B#18	gACggCACCCAgTTCgTgA		

Table III. HLA-DRB Primers and Probes for TaqMan Method

PROBE HLA-DRB: *5 ' FAM-CTTCgACAgCgACgTggiggAgT-L(TAMARA)PO_4 3'*

SPECIFICITY	5'PRIMER	5' SENSE 3'	3' PRIMER	5' ANTISENSE 3'
03011/021/03/05-08	5.03	TACTTCCATAACCAggAggAgA	3.03	TgCAgTAgTTgTCCACCCg
0301/04/06/08/10/11,1327	5.06	gACggAgCgggTgCggTA	3.48	CTgCACTgTgAAgCTCTCCA
0302/05,1109/20,1302/05/26/29/31,1402/03/09/13/19/24/27/30	5.03	TACCTTCCATAACCAggAggAgA	3.47	CTgCACTgTgAAgCTCTCAC
1116/20,1301/02/15/16/27/28/31	5.03	TACCTTCCATAACCAggAggAgA	3.10	CCCgCTCgTCTTCCAggAT
1303/04/12/13/21/30/32/33,1413	5.05	gTTTCTTggAgTACTCTACgTC	3.45	TgTTCCAgTACTCggCgCT
1109,1305/18/26,1427	5.03	TACCTTCCATAACCAggAggAgA	3.17	CCCgCCTgTCTTCCAggAA
1401/04/05/07/08/11/14/18/23/26/28	5.05 5.08	gTTTCTTggAgTACTCTACgTC AgTACTCTACgggTgAgTgTT	3.11	TCTgCAATAggTgTCCACCT
1109,1305/06/26,1402/06/09/13/17/29/30	5.03	TACCTTCCATAACCAggAggAgA	3.12	TCCACCgCggCCCgCC
0101-04	5.01	TTgTggCAgCTTAAgTTTgAAT	3.47 3.48	CTgCACTgTgAAgCTCTCAC CTgCACTgTgAAgCTCTCCA
0103	5.01	TTgTggCAgCTTAAgTTTgAAT	3.10	CCCgCTCgTCTTCCAggAT
1501-03/06	5.02	TCCTgTggCAgCCTAAgAg	3.01	CCgCgCCTgCTCCAggAT
16011/021/03/05/07/08	5.02	TCCTgTggCAgCCTAAgAg	3.02	AggTgTCCACCgCggCg
0102	5.01	TTgTggCAgCTTAAgTTTgAAT	3.08	CACTgTgAAgCTCTCCACAg
0801-06/09/10/12/14/16-19,1317,1415	5.08	AgTACTCTACgggTgAgTgTT	3.14 3.45	gCTgTTCCAgTACTCggCAT TgTTCCAgTACTCggCgCT
0401-22/24-27,1122,1410	5.04	gTTTCTTggAgCAggTTAAACA	3.47 3.48	CTgCACTgTgAAgCTCTCAC CTgCACTgTgAAgCTCTCCA
0701/03	5.07	CCTgTggCAgggTAAgTATA	3.79	CCCgTAgTTgTgTCTgCACAC
09012	5.09	gTTTCTTgAAgCAggATAAgTTT	3.79	CCCgTAgTTgTgTCTgCACAC
0308,1101-04/06-21/23-29/31	5.05	gTTTCTTggAgTACTCTACgTC	3.06	CTggCTgTTCCAgTACTCCT
0812,1201/02/04/05,1428	5.08	AgTACTCTACgggTgAgTgTT	3.08	CACTgTgAAgCTCTCCACAg
1001	5.10	CggTTgCTggAAAgACgCg	3.14	gCTgTTCCAgTACTCggCAT
0301-07/09/11,1301-02/05-11/14-16/18-20/22-25/27-29,1402-03/06/09/12/14/17/19-21/23/24/27/29/30	5.05	gTTTCTTggAgTACTCTACgTC	3.14	gCTgTTCCAgTACTCggCAT

Table IV. HLA-DQA1 Primer and Probes for TaqMan Method

PROBE: HLA-DQA1 *-TggACCTggAgAggAAggAgACTgCCT-L(TAMARA)$PO_4$3'*

SPECIFICITY	5' PRIMER	5' SENSE 3'	3' PRIMER	5' ANTISENSE 3'
0101/04	A-5'01	CATgAATTTgATggAgATgAgg	A-3'01	ATgATgTTCAAgTTgTgTTTTgC
0101/2/4	A-5'02	ACggTCCCTCTggCCAgTA	A-3'01	ATgATgTTCAAgTTgTgTTTTgC
0102/3	A-5'03	CATgAATTTgATggAgATgAgC	A-3'01	ATgATgTTCAAgTTgTgTTTTgC
0103	A-5'04	ACggTCCCTCTggCCAgTT	A-3'01	ATgATgTTCAAgTTgTgTTTTgC
0201	A-5'04	ACggTCCCTCTggCCAgTT	A-3'02	CAggATgTTCAAgTTATgTTTTAg
0301	A-5'05	TTCACTCgTCAgCTgACCAT	A-3'03	CAAATTgCgggTCAAATCTTCT
0302	A-5'06	TTCACTCgTCAgCTgACCAC	A-3'03	CAAATTgCgggTCAAATCTTCT
0401	A-5'07	ACCCATgAATTTgATggAgAC	A-3'04	CACATACCATTggTAgCAgCA
0501	A-5'02	ACggTCCCTCTggCCAgTA	A-3'05	AgTTggAgCgTTTAATCAgAC
0601	A-5'04	ACggTCCCTCTggCCAgTT	A-3'06	ggTCAAATCTAAATTgTCTgAgA
1 "A"	A-5'08	AgCCCTTgTggAggTgAAgA	A-3'07	AgTggTTggggCTCTggTTT
0104	A-5'09	gCCCTTgTggAggTgAAgg	A-3'07	AgTggTTggggCTCTggTTT

Table V. HLA-DQB1 Primers and Probes for TaqMan Method

PROBE: HLA-DQB02 *5' FAM-CCgAgAAgAgATCgCACgCTTCgACA-L(TAMARA)PO_4 3'*

SPECIFICITY	5' PRIMER	5' SENSE 3'	3' PRIMER	5' ANTISENSE 3'
0201	B-5'07	gTgCgTCTTgTgAgCAgAAg	B- 3'07	gCAAggTCgTgCggAgCT

PROBE: HLA-DQB03 5' FAM-CgAgAggAgTACgCACgCTTCgACAgC- L(TAMARA)PO_4 3'

SPECIFICITY	5' PRIMER	5' SENSE 3'	3' PRIMER	5' ANTISENSE 3'
0301	B-5'09	g ACggAgCgCgTgCgTTA	B- 3'09A	gTACTCggCgTCAggCg
0302	B-5'08	gACggAgCgCgTgCgTCT	B- 3'08	ggCTgTTCCAgTACTCgiCgg
0303	B-5'08	gACggAgCgCgTgCgTCT	B- 3'10	ggCTgTTCCAgTACTiggCgT

PROBE: HLA-DQB05 *5' FAM-ATAACCgAgAggAgTACgTgCgCTTCgAC-L(TAMARA)PO_4 3'*

SPECIFICITY	5' PRIMER	5' SENSE 3'	3' PRIMER	5' ANTISENSE 3'
0501	B-5'01	CggAgCgCgTgCgggg	B- 3'01	gCTgTTCCAgTACTCggCAA
0502	B-5'02	TgCggggTgTgACCAgAC	B- 3'02	TgTTCCAgTACTCggCgCT
0503	B-5'02	TgCggggTgTgACCAgAC	B- 3'03	gCggCgTC ACCgCCCgA
0504	B-5'12	gTgCggggTgTgACCAgAT	B- 3'02	TgTTCCAgTACTCggCgCT
0601	B-5'03	gCCATgTgCTA CTTCACCAAT	B-3'04	CACCgTgTCCAACTCCgCT
0602	B-5'04	CgTgCgTCTTgTgACCAgAT	B-3'0	5gCTgTTCCAgTACTCggCAT
0603	B-5'05	ggAgCgCgTgCgTCTTgTA	B- 3'05	gC TgTTCCAgTACTCggCAT
0604	B-5'06	CgTgTACCAgTTTAAgggCA	B- 3'06	gCAggATCCCgCggTACC
060	B-5'13	CgTgTACCAgTTTAAgggCC	B- 3'06	gCAggATCCCgCggTACC
0401	B-5'10	CACCAACgggACCgAgCT	B- 3'11	ggTAgTTgTgTCTgCA TACg
0402	B-5'11	CACCAACgggACCgAgCg	B- 3'11	ggTAgTTgTgTCTgCATACg

Table VI. Final Concentrations of Primers and Probes for each Locus.

	HLA-A	HLA-B	DRB	DQA1	DQB1
Specific Primers	0.25 µM	2.0 µM	0.25 µM	0.25 µM	0.25 µM
Specific Probe	50 nM	50 nM	100 nM	50 nM	50 nM
Control Primers	0.1 µM	0.8 µM	0.08 µM	0.075 µM	0.08 µM
Control Probe	50 nM	50 nM	100 nM	50 nM	50 nM

Quantitation of Cytokines by Competitive PCR

Patrizia Luppi and Massimo Trucco

Principle and Purpose

Cytokines are polypeptide humoral mediators promoting cell-to-cell interactions. They are generally secreted as the result of an immunologic response and are able to enhance or reduce target cell immunologic activities. Cytokines encompass hormone-like polypeptides called "lymphokines" if they are produced by lymphocytes or "monokines", if they are the product of monocytes or macrophages. Their activity is normally limited in time and space since they are physiologically effective only on targets located physically close to the cell that secretes them. A very large number of specific cytokines with different structures, molecular weights, activities and preferential targets have been described in the last few years.[1] Their most common names and specific characteristics are summarized in Table I.

Due to their particularly important immunologic functions, it is becoming important for both the scientist and clinician to be able to recognize their presence and to define and quantitate their expression. Although commercially available kits exist for the immunologic (i.e., via antibodies) detection of the expressed products, this expression can be optimally monitored by measuring cytokine specific mRNAs in the cell population under study. It is also important to be able to characterize even relatively small differences in the quantity of gene transcripts, in a limited number of cells constituting a homogeneous population. Conventional methods like Northern blot analysis or "dot blot" hybridization are frequently not sensitive enough for these very sophisticated purposes. The advent of the polymerase chain reaction (PCR)[2] made it possible to amplify individual mRNA templates once transformed by reverse transcriptase (RT) into cDNA. The main advantages of this so called RT-PCR technique[3] over traditional techniques includes a 3-4 order of magnitude higher sensitivity, and the possibility of simultaneously determining the amount of various mRNA molecules in the same sample. In this way, without laborious techniques, very precise quantitative results can then be obtained. Today, RT-PCR is generally considered the best available approach to detect mRNA of low abundance, in samples with low cell number or in a limited amount of tissue. Also, expression of each specific mRNA can be tested in cell populations where mixtures of different cytokines are simultaneously produced.

A major limitation of the original RT-PCR approach for cytokine determination has usually been the difficulty of generating absolute quantitative information. In fact, small variations in amplification efficiency may result in dramatic changes in product yields. It is the nature of the process itself that makes it prone to exponentially growing differences. Also, after a certain number of cycles, when the ingredients necessary to progress with the reaction become less available, the exponential growth of the generated product tends to plateau making it very difficult to evaluate differences in the levels of the target mRNA. Thus, the use of internal controls becomes mandatory to compare the efficiency of the PCR in different reactions and to avoid misinterpretations of the results. Two types of internal controls can be used: 1) an endogenous sequence that is normally present in the sample or 2) an exogenous fragment added to the PCR reaction. The theory behind the use of both types of internal controls is that the amount of amplified control can be separately measured at the end of the experiment. And, in turn, the amount of the control and of the target products at the end of the reaction are proportional to the relative amounts of the control and target templates present at the beginning of the reaction.

1. Quantitative PCR using an endogenous sequence as internal control.

The most frequently utilized internal controls are represented by so-called endogenous sequences that will be co-amplified with the target cDNA in the same PCR reaction. An endogenous control is a gene transcript independently expressed and invariant in the experiment, that is known to be normally expressed in all the various samples to be tested (e.g., a so-called "housekeeping gene"). Two sets of primers are used in the same PCR reaction — one set specific to the target gene cDNA and the other set specific to the housekeeping gene. It is imperative to know that the endogenous control is present at constant levels throughout the series of samples to be compared. One example of the successful utilization of this type of internal control is the amplification of a segment of the gene encoding the constant part of the alpha chain (Cα) of the T-cell receptor molecule when the repertoire of the various variable β (Vβ) chain gene segments is being tested. The same α chain cDNA segment, amplified in the same tube of each of the Vβ segments, will then be used as a common denominator to quantitatively compare the expression of all of the various Vβ chain gene families in a specimen (Figure 1).[4] Other genes successfully utilized for this purpose are actin,[5,6] β_2 microglobulin,[5] dihydrofolate reductase (DHFR)[7] and glyceraldehyde phosphate dehydrogenase (GAPDH).[8] One limit of this method is that the reference and target gene transcripts need to be expressed at similar levels. If the reference and the target genes are not present at comparable levels in the RNA sample, analysis of PCR products must be assayed at several different numbers of cycles. This is to ensure that analysis of both genes is performed only during the exponential phase of the amplification, when the respective amounts of the products are proportional to their amounts in the starting sample.[9] Another limit of using endogenous controls in quantitative PCR is the not-too-remote possibility that multiple sets of primers may interfere with the amplification of either or both of the target genes.

2. Quantitative PCR using an exogenous sequence as internal control.

This approach features the co-amplification of a DNA fragment from an exogenous source as the internal standard, in the same tube where the specimen is tested. There is a significant advantage in using an exogenously-added sequence instead of an endogenous gene transcript as the internal control. By using an exogenously added sequence, the initial amount of standard used in the PCR reaction is precisely known. This makes it possible to determine the level of target cDNA present in the sample that corresponds to the initial mRNA levels. The internal standard is represented by either a homologous or non-homologous[10,11] DNA fragment engineered to contain the same primer template sequences as the target DNA, but able to generate a PCR product of a different size. In this type of amplification, only one set of primers is used to amplify both the target cDNA and the other DNA fragment (i.e., the exogenous control). In this context, primer concentrations, their binding affinity, and Taq polymerase activity are expected to be exactly the same for both the target cDNA and the internal control added to the same tube.

Thus, amplification takes place in a truly "competitive" fashion, where the control DNA fragment competes with the target cDNA for the same PCR reagents and, therefore, for amplification as well (competitive PCR). In a competitive PCR, serial dilutions of either the target sequence or the internal control sequence are made and a constant amount of the other component is added to each reaction. Differences in size of the two co-amplified products (or the availability of a certain restriction site in only one of the two amplified segments), allows the two products to be distinguished from each other (i.e., target segment versus competitor segment) after being separated in an agarose gel. The assumption here is that the size difference of the competitor fragment and the target template is small enough that they will amplify with equal efficiency. An important advantage of the competitive PCR is that, because the ratio of target to control remains constant during the amplification, it is not necessary to obtain data before the reaction reaches the plateau phase of the amplification. Because of the ability to make a reliable and accurate quantitative analysis of gene transcripts, the competitive PCR approach is useful for assessment of cytokine mRNA in many different types of specimens. Hereafter, we will describe two competitive PCR approaches — one using homologous, the other using non-homologous DNA fragments as exogenous internal control — for cytokine mRNA measurement.

a. Competitive PCR using homologous DNA competitor fragments or a complementary RNA (cRNA) segment

The first attempt in this direction involved the use of a genomic fragment of the target gene.[12,13] The genomic fragment was chosen so that it contained a small intron, which allowed the amplification of a segment of DNA longer than the target cDNA. An alternative approach used a target cDNA modified to contain a unique restriction site. After digestion of the amplified product with the appropriate restriction enzyme, the smaller segments (i.e., the control) could easily be distinguished from the still-intact segment from the target sequence. For quantitative determination, each different cytokine needed a particular cDNA segment as an internal control. However, a more versatile approach uses a competitive template consisting of a single synthetic DNA or a mRNA segment that encompasses the primers specific for all the various cytokines to be tested.[14,15] In this approach, the control is a DNA segment cloned in a vector and containing, in a tandem array, 5′ and 3′ primer sequences specific for the various cytokines[11] (Figure 2). Alternatively, a complementary RNA (cRNA) segment can be obtained by transcription of a similar linear array of these sequences.[12] To allow the generation of the desired RNA, the plasmid containing the series of primers, also features a specific promoter (e.g., T7 polymerase) for DNA-dependent RNA polymerase which then provides a template for cRNA synthesis. This cRNA-based control approach offers an additional advantage over the approach that utilizes a DNA control in that the possible variations due to differences in the efficiency of the RT step are eliminated. Control and target RNA are processed for the conversion into cDNA simultaneously in the same test tube (Figure 3). Furthermore, different known molar concentrations of competitor cRNA (or cDNA) can be added into a series of RT-PCR (or PCR) reaction tubes containing a fixed amount of target template. The co-amplified products can then be compared to each other on a gel. The amount of competitor yielding equimolar amounts of target product indicates the initial target gene amount.[12,13]

Another advantage of this method is the fact that since the initial ratio of target-to-competitor template remains constant throughout the amplification, it is not critical to obtain data during the exponential phase of the reaction. In many of the more recently prepared constructs only the specific primer sequences are taken from the original template while irrelevant intervening sequences are added at will to generate segments easily distinguishable by size from the target segment to be tested. These methods definitively yield reliable quantitative information with an accuracy that allows the precise assessment of the quantity of target RNA present in the initial sample.

b. Competitive PCR using non-homologous DNA competitor fragments

Non-homologous DNA fragments of a desired size may be engineered to share the same primer template sequences so to be usable in a competitive PCR as exogenous internal standards. One example of non-homologous DNA fragments is the viral oncogene v-*erb* B gene[16] to which specific primer templates have been added creating the so-called PCR MIMICS, because they "mimic", or closely imitate, the primer binding and amplification characteristics of the target.[11] Amplified genomic DNA segments from other species represents another form of homologous internal standards.[17] These DNA fragments are commercially available but must be used with PCR primer sets provided by the manufacturer.

Specimen

Cytokine assessment can be performed on any type of tissues where the presence of mononuclear cells are suspected or documented. For tissue samples, either biopsy or surgical specimens can be utilized for cytokine measurement. Special precautions must be taken to avoid RNA degradation in the samples. For this purpose, proper handling of source material and the timing of tissue collection are very important. Tissue specimens must be collected in sterile polypropylene tubes as soon as possible after surgery and immediately processed. If RNA extraction is not performed soon after the collection of the tissue, samples must be immediately frozen at –80°C. However, it is preferable to store tissue samples after being homogenized in the ready-to-use reagent for RNA isolation (see PROCEDURE). After homogenization, and before addition of chloroform, samples can be successfully stored at –80°C for at least one month. Alternatively, it is possible to immediately proceed to RNA extraction (see PROCEDURE). Cytokine mRNA assessment can be also performed on peripheral blood mononuclear cells (PBMC) isolated from heparinized venous blood by Ficoll-Hypaque density gradient centrifugation or from cultured cells. RNA extraction on freshly isolated PBMC should be performed soon after the blood drawn. Storage of blood samples at room temperature for several hours reduces the recovery of RNA.

To increase cytokine production by PBMC, these cells can be cultured with different types of mitogens, such as phytohaemagglutinin (PHA) that preferentially stimulates T cells, and lipopolysaccharide (LPS) that preferentially stimulates macrophages.[18] The same precautions used to avoid RNA degradation in isolation from tissue samples are also required for cell samples. Cells must be spun down in a polypropylene tube to form a cell pellet and then immediately stored at –80°C if RNA isolation is not immediately performed. However, storage of cell pellets homogenized in the ready-to-use reagent for RNA extraction is preferable (see PROCEDURE).

Cytokine mRNA analysis performed on tissue samples or in cells that are inappropriately collected or stored will result in RNA degradation which will affect gene transcript signals and final results.

Reagents and Supplies

A. Competitive PCR using homologous DNA competitor fragments or a complementary RNA (cRNA) segment

1. RNase-free pipette-tips, ART®, Molecular Bio-Products;
2. Siliconized RNase-Free microfuge tubes 1.5 ml size, Rnase-Free, Ambion #12450;
3. Thin-walled microtubes with attached cap 0.2 ml, USP #PCR-02Y, or thin-walled 0.2 or 0.5 ml PCR tubes;
4. Disposable gloves, Pharmaseal #8877;
5. Plasmid, containing control sequence(s);
6. Specific restriction enzyme(s);
7. Proteinase K solution for removal of contaminating RNase from DNA plasmid preparations, Ambion # 2546. Store at –20°C not in a frost-free freezer;
8. 10% SDS. Store at room temperature;
9. Phenol/$CHCl_3$, Ambion # 9730. Store at 4°C or at 20°C as small aliquots;
10. *In vitro* transcription kit for synthesis of capped RNAs, mMESSAGE mMACHINETM, Ambion #1344. Store at 20°C;
11. DEPC-Treated H_2O, RNase-Free, DNase-Free, Ambion #9920. Store at room temperature;
12. See reagents for RNA extraction;
13. Isolation and purification of intact mRNA, Dynabeads® mRNA DIRECT kit, Dynal # 610.11. Store at –20°C;
14. Columns for radiolabeled DNA purification, Quick SpinTM Columns, G-25 Sephadex® Columns, Boehringer Mannheim #100400;
15. PCR primers for use in detection and analysis of human, mouse, or rat mRNA. Store at –20°C;
16. 10x PCR buffer, Perkin Elmer # N808-0153. Store at –20°C;
17. 25mM $MgCl_2$, Perkin Elmer # N808-0153. Store at –20°C;
18. 10mM Premixed deoxynucleotide solution for use in the PCR, PCR Nucleotides Mix, Boehringer Mannheim #1581295. Store at –20°C.
19. Taq DNA polymerase, AmpliTaq® Perkin Elmer # N808-0153. Store at –20°C;
20. Autoclaved, distilled H_2O;

B. Competitive PCR using non-homologous competitor DNA fragment

1. Pipette-tips, ART®, Molecular Bio-Products;
2. Thin-walled microtubes with attached cap 0.2 ml, USP #PCR-02Y or thin-walled 0.2 or 0.5 ml PCR tubes;
3. Disposable gloves, Pharmaseal #8877;
4. PCR primers for use in detection and analysis of human, mouse, or rat mRNA, RT-PCR Amplimer Sets, Clontech. Store at –20°C in a constant-temperature (not frost-free) freezer;
5. Non-homologous DNA fragments for use as competitive internal standards in PCR, PCR MIMICsTM, Clontech. Store at –20°C in a constant-temperature (not frost-free) freezer;
6. DNA polymerase for amplification of cDNA, Advantage cDNA Polymerase kit, Clontech #8417-1. Store at –20°C.
7. 10mM Premixed deoxyucleotide solution for use in the PCR, PCR Nucleotides Mix, Boehringer Mannheim #1581295. Store at –20°C;
8. GelStar® nucleic acid gel stain, FMC BioProducts #50535. Store at -20°C;
9. Polaroid Type 55 Film.
10. Autoclaved, distilled H_2O;

C. RNA Extraction

1. Sterile disposable polypropylene tubes 15 ml, Corning # 25319-15 or sterile disposable polypropylene tubes 50 ml, Fisher Scientific # 05-539-6;
2. Siliconized RNase-Free microfuge tubes 1.5 ml size, Rnase-Free, Ambion #12450;
3. RNase-free pipette-tips, ART® Molecular Bio-Products;
4. Disposable gloves, Pharmaseal #8877;
5. RNA extraction kit, TRIzol® Reagent, GIBCO BRL #15596-026. Store at 2° to 8°C;
6. Chloroform: $CHCl_3$, HPLC reagent grade, J.T Baker #9175. Store at room temperature;
7. Isopropanol: C_3H_8O, 99+% for molecular biology, SIGMA #I-9516. Store at room temperature;
8. 75% Ethyl alcohol (prepared using DEPC-treated water). Store at room temperature;
9. DEPC-Treated H_2O, RNase-Free, DNase-Free, Ambion #9920. Store at room temperature;
10. MessageCleanTM kit, for removal of DNA contaminant from RNA, GenHunter Corporation #M601. Store at –20°C.
    Note: All the reagents and supplies should be stored in a separate cabinet and only used for RNA isolation.

D. Reverse-Transcriptase reaction (RT-PCR)

1. RNase-free pipette-tips, ART®, Molecular Bio-Products;
2. Disposable gloves, Pharmaseal #8877;
3. Thin-Walled PCR tubes 0.5 ml size, RNase-Free, Ambion #12250.
4. RT-PCR kit, SuperscriptTM preamplification system for first strand cDNA synthesis, GIBCO #18089-011. Store at –20°C.
5. Glycogen, special quality for molecular biology, Boehringer Mannheim #901-393. Store at –20°C;
6. DEPC-treated H_2O, RNase-Free, DNase-Free, Ambion #9920. Store at room temperature.

E. Amplification of the target cDNA

1. Pipette-tips, ART®, Molecular Bio-Products;
2. Thin-walled microtubes with attached cap 0.2 ml, USP #PCR-02Y, or thin-walled 0.2 or 0.5 ml PCR tubes;
3. Disposable gloves, Pharmaseal #8877;
4. Two amplification primers specific for the target cDNA. For example, amplification of the glyceraldehyde phosphated dehydrogenase (GAPDH);
5. 10x PCR buffer, Perkin Elmer # N808-0153. Store at –20°C;
6. 25mM $MgCl_2$, Perkin Elmer # N808-0153. Store at –20°C;
7. 10mM Premixed deoxyucleotide solution for use in the PCR, PCR Nucleotides Mix, Boehringer Mannheim #1581295. Store at –20°C;
8. Taq DNA polymerase, AmpliTaq® Perkin Elmer # N808-0153. Store at –20°C;
9. Autoclaved, distilled H_2O.

Instrumentation/Equipment

1. Automatic pipettes capable of dispensing 1 to 20 µl, 20 to 200 µl, and 500 to 1000 µl;
2. Programmable Perkin-Elmer DNA Thermal Cycler (Model 9600);
3. Microcentrifuge capable of generating a relative centrifugal force of 14,000xg;
4. 37°C, 42°C, 70°C water baths or heat blocks;
5. Equipment for gel electrophoresis;
6. UV Transilluminator;
7. Analytical image device to scan ethidium bromide stained gels; Densitometer, Molecular Dynamics;
8. Analytical image device to scan radiolabeled gels; Molecular Dynamics Phosphorimager, SI;
9. Power homogenizer (Polytron).

Optional

Hybridization probes to confirm specificity of amplifies products;
Sequence apparatus for determination of sequences of obtained amplifications, ABI 377 DNA Sequencer (Applied Biosystems).

Calibration

For competitive PCR using non-homologous DNA competitor fragments, see PROCEDURE B2a.

Quality Control

1. Controls

a. Control for the presence of DNA contamination of the RNA sample.
   Usually this control is not necessary because the oligonucleotides used in the cytokine amplification have been chosen to amplify only spliced mRNA.

b. Control for the synthesis of the first strand of cDNA.
   A control RNA, as a template for reverse transcription, is usually included in the RT-PCR kit. The efficiency of the reaction can be determined following the manufacturer's instructions. The efficiency of the first strand cDNA syn-

thesis can also be checked by amplification of the newly synthesized cDNA for a "housekeeping gene", as described in PROCEDURE (paragraph B1c).

c. Negative control for competitive PCR for specific cytokine amplification. A negative control, composed of distilled water, is used in every amplification in place of the template cDNA from RT.

2. Reagents-Basic Guidelines

a. Do not open more than one container of a reagent or chemical at any one time unless the one currently in use is suspect.
b. Note the date of any change of brand or method of preparation.
c. For RNAse-free materials that can be stored at room temperature, store them in a separate cabinet and only use them when working with RNA.

3. Reagents-Commercial Materials

a. Upon receipt of supplies, mark the receiving and expiration date on the label before storage.
b. When opened, label with date, technologist's initials, expiration date.

4. Reagents-Prepared in the Laboratory

Label properly (including working bottles) with:

- Reagent name
- Concentration and/or pH
- Date of preparation
- Technologist's initials
- Date of expiration

5. Oligonucleotides for cytokine-specific amplification

a. Synthetic sequence specific oligonucleotide probes (SSOPs) and primers must be carefully quantitated, aliquoted and stored for long term usage.
b. Each newly prepared primer set or probe is tested with the competitive template prior to use. Amplifications should be monitored by gel electrophoresis to detect ethidium bromide stained bands from the appropriate cells and by hybridization with the appropriate probes, designed to detect nonspecific amplification. Primers at the appropriate concentration are aliquoted and stored frozen, or in small aliquots at 4°C.

6. Procedure to Store Oligonucleotide Primers

NOTE: Oligonucleotides can be either purchased or synthesized in house using an ABI 493 DNA/RNA Synthesizer. They are usually received suspended at various concentrations. Carefully note the concentrations on the paperwork provided with each synthesis.

NOTE: Perform all dilutions in laminar flow hood.

a. Primers are diluted directly to appropriate concentrations (20 pm/µl). Store stocks at -20°C and working dilutions at 4°C.
b. Plasmid stocks are prepared at 5 ng/µl and stored at -20°C.

7. Method for Performing Wipe Tests:

NOTE: This procedure should be done weekly and should include a minimum of 10 samples, including such areas as lab benches, laminar flow hoods, work surfaces or centrifuges.

a. Wear gloves and lab coat.
b. Decontaminate forceps by wiping them with 10% bleach made with ddH_2O and then rinsing the forceps in ddH_2O.
c. Wet a 1.0 cm diameter disk of Whatman 3 mm paper in ddH_2O using a pair of decontaminated forceps.
d. Wipe wet filter paper over an approximate 10 cm square area.
e. Place filter paper disk in a 1.5 ml microfuge tube with 400 µl of ddH_2O and briefly vortex.
f. Incubate at 56°C for 1 hour.
g. Quick spin to force the filter paper to the bottom of the tube.
h. Use 50 µl of the wipe test sample liquid in a 100 µl PCR reaction.[9] Amplify the wipe test samples using the cytokine-specific primers following the lab's standard PCR protocol. Use the same number of amplification cycles as routinely used for donor samples.
i. After PCR, electrophoresed 10-20 µl of each test sample in an agarose gel stained with ethidium bromide.
j. If any areas are found to be contaminated, clean area thoroughly with 10% bleach, then retest. DNA prep area must test negatively before work can resume.

8. Instrument Care and Quality Control

NOTE: All temperatures are monitored for accuracy with calibrated alcohol thermometers independently of the instrument's internal temperature reading.

a. **Laminar Flow Hoods:** The top work areas should be cleaned with 70% ethanol or 10% bleach solution after every use, followed by at least 20 minutes of UV The airflow is checked and serviced once a year by a certified serviceman.
b. **Refrigerators/freezers:** Temperatures should be recorded daily. Temperatures should be accurate within ± 2°C for refrigerators and ± 3°C for freezers.
c. **Centrifuges:** Centrifuges are cleaned after each use with 70% ethanol or 10% bleach solution.

d. **Water baths:** Temperatures of 37°C, 42°C and 70°C water baths should be recorded prior to each use. Water levels are checked daily. Shaking baths are cleaned monthly and other water baths are cleaned as needed. Temperatures of water baths should be accurate within ± 1°C.
e. **Pipettors:** Pipettors should be checked for accuracy and reproducibility every 6 months. Pipettors are sent out for calibration and repair as needed.
f. **Incubators:** Temperatures of incubators should be recorded before each use. Incubator temperature should be accurate within ± 1°C.
g. **Thermal cyclers:** Thermal cyclers should be cleaned weekly with 50% ethanol and monthly with 10% bleach solution. Heater/chiller and verification of temperature calibration tests should be performed monthly. Temperature accuracy and cycle time reproducibility tests should be performed every six months. Consult thermal cycler user manual for protocols on performing the diagnostic tests.

Procedure

A. Competitive PCR using homologous DNA competitor fragments or a complementary RNA (cRNA) segment

1. *In Vitro* Transcription of RNA Template

In the original method described by Wang et al.[14] the different control cRNA segments are obtained by taking advantage of the features of the Okayama-Berg vector in which the sequence containing the various 5′ and 3′ primers was cloned. This new construct, called pAW108, can be used as a template for transcription by the T7 polymerase since it contains on the one side the T7 polymerase promoter (Figure 3). The preparation of the DNA template for *in vitro* transcription is relatively straightforward, requiring clean DNA, restriction enzymes and other ribonuclease-free reagents. The basic steps are as follows:

a. **Preparation of the linearized template.** The plasmid DNA is generally digested using a restriction enzyme to make a template that will generate transcripts of a defined size. The pAW180 vector should be digested with Bam HI that cleaves on the 3′ side of the insert (Figure 3);
b. **Eliminating the contaminating Rnase.** All contaminating RNAse must be removed before attempting to use the plasmid DNA as a template for transcription. This can be done by treating the linearized template with Proteinase K (100-200 µg/ml) and SDS (0.5%) for 1 hour at 50°C, followed by phenol/$CHCl_3$ extraction and ethanol precipitation using RNase-free reagents and all the precautions necessary for RNA extraction;
c. ***In vitro* transcription reaction for synthesis of RNA.** One microgram of a linearized plasmid containing 0.5-5kb insert is generally optimal for a 20 µl transcription reaction using mMESSAGE mMACHINETM kit following manufacturer's instructions.

Briefly:
Add the following amounts of the indicated reagents in the order shown to a 1.5 ml Rnase-free microcentrifuge tube at room temperature:

Component	Sample
Nuclease-free dH_2O	to 20 µl
10x Reaction Buffer	2µl
2x Ribonucleotide Mix	2 µl
1 µg linearized template DNA	n µl
10x Enzyme Mix	2 µl
Final volume	**20 µl**

- Incubate the reaction at 37°C in an incubator for 1 hour;
- After the transcription reaction is complete, the template DNA may be degraded by the addition of 1 µl of RNase-free DNase I and further incubated at 37°C for 15 minutes;

d. **Purification of the AW180 cRNA**

The resulting AW180 cRNA product is purified by oligo (dT), thanks to the polyadenylated sequence present on the 3′ end of the construct. The isolation of highly purified intact mRNA is achieved using DynabeadsR mRNA DIRECT kit following the manufacturer's instructions. Briefly, for up to 10 µg of RNA:

1) Adjust the volume of your reaction to 20 µl (for up to 10 µg of RNA) with distilled DEPC-treated H_2O;
2) Remove 40 µl of resuspended Dynabeads® Oligo (dT)25 from the product tube. Dispense the beads into a 0.5 ml tube standing in the apposite Dynal MPCR-E-1 magnet. After 30 seconds remove the supernatant and wash once with 20 µl 2x binding buffer;
3) Transfer the tube to another rack and add 20 µl 2x binding buffer;
4) Heat RNA in 20 µl of DEPC-treated H_2O to 65°C for 2 minutes to disrupt secondary structures;
5) Add the RNA to the bead solution. Mix gently. Let stand to hybridize for 5 minutes at room temperature;
6) Place the tube in the Dynal MPC®-E-1 magnet for 30 seconds and remove the supernatant;
7) Remove the tube and wash 3 times with 40 µl of washing buffer. Be sure to remove all the supernatant after the final washing step;
8) Add desired amount (down to 5 µl) of elution buffer. Heat to 65°C for 2 minutes, place the tube immediately into the Dynal MPC®-E-1 magnet. Transfer the eluted mRNA to a new Rnase-free tube. The Dynabeads® Oligo $(dT)_{25}$ are bound to the magnet.

9) If the eluted mRNA is not used immediately, it should be stored frozen at 80°C;
10) Measure the absorbance at 260 nm of the mRNA and calculate the number of AW180 cRNA molecules (see in CALCULATIONS);
11) A recovery of 20 µg of RNA per µg of template is expected.

e. **Conducting competitive reverse-transcription (RT)-PCR:**
In this procedure, different dilutions (e.g., 1.77 x 10^2-10^6 molecules of AW180 cRNA) of the control are reverse transcribed in cDNA together with a fixed quantity of the target RNA (e.g., 1 µg of total cellular RNA) in the same tube. The cellular RNA (target) is normally isolated using TRIzoll® Reagent as will be described in B (Isolation of total RNA) and RT-reaction can be performed using SUPERSCRIPT TM Pre-amplification system for first cDNA synthesis (GIBCO, BRL), as will be described in B (First strand cDNA synthesis from total RNA).

2. Human Cytokine Specific Oligonucleotides
The oligonucleotide primers used for amplification in Wang's method are shown in Table 2. They can be labeled with [g-^{32}P] by using a polynucleotide kinase to make a more sensitive test than the one in which bands are visualized with ethidium bromide. Unincorporated nucleotides can be removed on a Quick SpinTM Columns.

3. Cytokine-specific Amplification
Two microliters of the cDNA is then used in a cytokine-specific amplification. Add the following to a 0.2 or 0.5 ml thin walled, PCR tube:

Component	Volume	
10x PCR Buffer	5 µl	
25mM $MgCl_2$	3 µl	
10mM dNTPs	1 µl	
5′-cytokine specific primer (5 µM)	1 µl	(0.1 µM)
3′-cytokine specific primer (5 µM)	1 µl	(0.1 µM)
(or 1 x 10^6 cpm of ^{32}P-end labeled primer)		
cDNA (from the first strand reaction)	2 µl	
Autoclaved, distilled H_2O	36 µl	
Taq DNA polymerase (2 to 5 units/µl)	1 µl	
Final volume	50 µl	

Perform 30-35 cycles of PCR with the following conditions:
94°C x 30 sec;
55°C x 30 sec;
72°C x 1 min.

After PCR, electrophorese 10 µl of each cytokine-specific PCR reaction mixture in an 8% polyacrylamide gel in 0.5x Tris borate/EDTA buffer. Stain by adding ethidium bromide (0.5 µg/ml) to the gel buffer. Quantification of the amount of the target material in the PCR sample is determined as described in CALCULATIONS.

B. Competitive PCR using non-homologous DNA competitor fragment

1. Preparation of Target Sequence

a. **Isolation of total RNA.**
The use of high quality RNA is critical for successful cDNA synthesis because it dictates the maximum amount of sequence information that can be converted into cDNA. Thus, it is important that RNA is not degraded by ribonucleases and is absolutely free from contaminants. Proper handling of source material and rapid, efficient cell disruption in denaturing solutions usually prevent adventitious introduction of ribonucleases and other reagents. These are the most critical factors in the extraction of intact RNA. Isolation of total RNA requires less time and manipulation than purification of poly(A) RNA, and therefore, total RNA is typically used whenever possible. Total RNA can be isolated by many methods. The most common and successful methods are modifications of the original guanidinium thiocyanate method described by Chomczynski and Sacchi.[19] Several companies now offer kits for RNA extraction. Protocols in the instruction manuals for each kit provide tips for RNA isolation from many different tissue sources.

The TRIzoll® Reagent (GIBCO BRL) which allows the isolation of RNA from very little starting material (as little as 1x10^3 cells) and is advantageous in applications where only small amounts of cellular material are available has already been successfully used.[20] The most critical steps in RNA isolation are the following:
1) HOMOGENIZATION
For tissues:
Homogenize tissue samples in 1 ml of TRIzoll® Reagent per 50-100 mg of tissue using a power homogenizer (Polytron);
For cells grown in monolayer:
Lyse cells directly in a culture dish by adding 1 ml of TRIzoll® Reagent to a 3.5 cm diameter dish, and passing the cell lysates several times through a pipette. The amount of the TRIzoll® Reagent added is based on the area of the culture dish (1 ml per 10 cm^2) and not on the number of cells present.

For cells grown in suspension or Ficoll-Hypaque extracted PBMC:
Pellet the cells by centrifugation. Lyse them in TRIzoll® Reagent by repetitive pipetting (use 1 ml of the Reagent per $5x10^6$ to $1x10^7$ of animal cells). Washing cells before addition of TRIzoll® Reagent should be avoided to minimize mRNA degradation.

2) PHASE SEPARATION
- Transfer 1 ml of the homogenized solution to a 1.5 ml RNase-free tube;
- Incubate the homogenized samples at room temperature for 5 minutes to permit the complete dissociation of nucleoprotein complexes.
- At this point, and before the addition of chloroform, samples can be stored at –80°C for at least one month. Alternatively, RNA isolation must be performed;
- Add 0.2 ml of chloroform per 1 ml of TRIzoll® Reagent. Cap sample tubes securely;
- Shake tubes vigorously by hand for 15 seconds and incubate at room temperature for 2-3 minutes;
- Centrifuge the samples at no more than 12,000 x g for 15 minutes at 4°C. Following centrifugation, the mixture separates into a lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA remains exclusively in the aqueous phase. The volume of the aqueous phase is about 60% of the volume of TRIzoll® Reagent used for homogenization.

3) RNA PRECIPITATION
- Transfer the aqueous phase to a fresh 1.5 ml tube;
- Precipitate the RNA by mixing with isopropanol (use 0.5 ml isopropanol per 1 ml of TRIzoll® Reagent used for the initial homogenization);
- Incubate samples at room temperature for 10 minutes and centrifuge at no more than 12,000 x g for 10 minutes at 4°C. The RNA precipitate, forms a gel-like pellet on the side and bottom of the tube;

4) RNA WASH
- Remove the supernate. Wash the RNA pellet once with 75% ethanol, adding at least 1 ml of 75% ethanol per 1 ml of TRIzoll® Reagent used for the initial homogenization;
- Mix the sample by vortexing and centrifuge at no more than 7,500 x g at 4°C.
- The RNA can be stored conveniently as an ethanol precipitate at –20°C or at –80°C until use.

5) REDISSOLVING THE RNA
- At the end of this procedure, briefly dry the RNA pellet (air dry or vacuum-dry for 5-10 minutes). Do not dry the RNA by centrifugation under vacuum. It is important not to let the RNA pellet dry completely as this will decrease its solubility. Partially dissolved RNA samples have an $A_{260/280}$ ratio of <1.6;
- Dissolve RNA in DEPC-treated H_2O and incubate 10-15 minutes at 55° to 60°C. RNA can be stored in aqueous solution at –80°C for at least up to one year without any appreciable deterioration. Repeated freeze-and-thaw cycles should be avoided.

b. **First strand cDNA synthesis from total RNA**

The isolated total RNA is then used in a reverse transcription reaction (RT) for the first strand synthesis of cDNA. Numerous commercial kits are now available for cDNA synthesis. We have successfully used[20] SUPERSCRIPT™ Pre-amplification system for first strand cDNA synthesis (GIBCO BRL) following manufacturer s instructions. Briefly, Prepare RNA/primer mixtures in sterile, RNase-free 0.5ml tubes as follows:

Component	**Sample**
DEPC-H_20	to 12 µl
1 to 5 µg total RNA	n µl
oligo (dT) (0.5 µg/µl)	1 µl
Final volume	**12 µl**

- Incubate each sample at 70°C for 10 minutes and incubate on ice for at least 1 minute;
- Prepare the following reaction mixture, adding each component in the indicated order (prepare the reaction mixture for n+1 samples):

Component	**Each Reaction**
10X PCR Buffer	2 µl
25 mM $MgCl_2$	2 µl
10 mM dNTP mix	1 µl
0.1 M DTT	2 µl
Final volume	**7 µl**

- Add 7 µl of the reaction mixture to each RNA/primer mixture, mix gently, and collect by brief centrifugation;
- Incubate at 42°C for 5 minutes;
- Add 1 µl of SUPERSCRIPT II RT to each tube, mix, and incubate at 42°C for 50 minutes;
- Terminate the reactions at 70°C for 15 minutes. Chill on ice;
- Collect the reactions by brief centrifugation.
- Add 1 µl of RNAase H to each tube and incubate for 20 minutes at 37°C;
- The newly synthesized cDNA (20 µl) must be stored at –20°C until use.

c. **Checking the efficiency of the first strand cDNA synthesis by amplification of the target cDNA.**
After the RT reaction, it is usually preferable to check the efficiency of the reaction by amplifying the cDNA for a gene that is known to be normally expressed in all the samples to be tested (a so-called "housekeeping gene"). For this purpose it can be used the GAPDH gene. Primers pairs for GAPDH amplification are as follows: sense (5′) TGA AGG TCG GAG TCA ACG GAT TTG GT (3′) and antisense (5′) CAT CTG GGC CAT GAG GTC CAC CAC (3′). Use only 10% of the first strand reaction for PCR (2 µl).

Add the following to a 0.2 or 0.5 ml thin walled, PCR tube:

Component	Volume
10x PCR buffer	10 µl
25mM $MgCl_2$	6 µl
10mM dNTP mix	2 µl
5 – GAPDH amplification primer (20 µM)	1 µl
3 – GAPDH amplification primer (20 µM)	1 µl
cDNA (from the first strand reaction)	2 µl
Autoclaved, distilled H_2O	77 µl
Taq DNA polymerase (2 to 5 units/µl)	1 µl
Final volume	**100 µl**

– Mix gently and incubate at 94°C for 3 minutes;
– Perform 30-35 cycles of PCR with the following conditions:
94°C x 20 sec;
58°C x 30 sec;
72°C x 30 sec.

Analyze 20 µl of the amplified sample using agarose gel electrophoresis. Amplification of the cDNA for the G3PDH gene gives a bright, sharp single 983-bp band.

2. Conduct Competitive PCR

For cytokine mRNA assessment, the PCR MIMICs[21] developed at CLONTECH, can be used in conjunction with corresponding RT-PCR Amplimer Sets. The theory behind the use of heterologous competitor fragments (i.e., PCR MIMICs) in quantitative PCR has been outlined in another section of this manual (see PRINCIPLE/PURPOSE). Basically, the principle is that if the competitor fragment and the target sequence amplify with the same efficiency, the initial ratio of target to standard is equal to the ratio of their amplification products. PCR MIMICs are expected to have similar amplification efficiencies as their corresponding target fragments and compete with them for the same primers in the same reaction. By knowing the amount of PCR MIMICs added to the reactions, it is possible to determine the amount of the target template, and thus the initial mRNA levels (Figure 4). This is achieved with two series of amplifications: a preliminary and then a fine-tuned competitive PCR amplification.

a. **Preliminary competitive PCR amplification**
First, a constant amount of the experimental target cDNA (from RT) is combined with serial dilutions of the PCR MIMICs following manufacturer's instructions. The manufacturer generally also provides a positive control target cDNA. A preliminary titration is performed using ten-fold dilutions of the PCR MIMIC stock solution which is provided by the manufacturer. Based on the results obtained from this amplification, a two-fold MIMIC serial dilution is set up for the precise quantitative PCR. Briefly:

(1) *Dilute the target cDNA:* The target cDNA from RT has to be appropriately diluted with distilled H_2O and then used for cytokine specific amplification. Generally, cDNA from peripheral blood mononuclear cells (PBMC) can be diluted in the proportion of 1:2 or 1:3 with distilled H_2O. cDNA synthesized from tissue-infiltrating lymphocytes can be diluted in the proportion of 1:1 or 1:2 with distilled H_2O depending on the degree of suspected or observed inflammation. The diluted cDNA is used for the specific quantification of the different cytokines;\

(2) Make the ten-fold MIMICs dilutions: Label eight 0.5 ml tubes M1-M8. Add 9 µl of MIMIC dilution solution to each tube. The diluting solution is made in TE buffer (10mM Tris-HCl, pH 7.5; 0.1mM EDTA) containing 10 µg/ml glycogen, nucleic acid grade.

Prepare the following ten-fold serial dilution stock solutions:

Concentration (attomole/µl)*	Tube Label	
100	M_0	MIMIC stock solution provided
10	M_1	Add 1 µl M^0, mix and change pipette tip
1	M_2	Add 1 µl M_1, mix and change pipette tip
10^{-1}	M_3	Add 1 µl M_2, mix and change pipette tip
10^{-2}	M_4	Add 1 µl M_3, mix and change pipette tip
10^{-3}	M_5	Add 1 µl M_4, mix and change pipette tip
10^{-4}	M_6	Add 1 µl M_5, mix and change pipette tip
10^{-5}	M_7	Add 1 µl M_6, mix and change pipette tip
10^{-6}	M_8	Add 1 µl M_7 and mix

* attomole = 10^{-18} moles

The dilution series can be stored at –20°C but multiple freeze-thaw cycles should be avoided.

(3) Set up six new tubes for PCR;

(4) Add to a tube for each dilution:

2 µl	cDNA from RT
2 µl	one dilution of the MIMICs (i.e., M2 to M7)
46 µl	PCR Master mix
50 µl	**Final reaction volume**

PCR Master Mix

Prepare enough PCR Master mix for each experiment plus an extra sample (i.e., if 6 tubes, makes mix for 7) using the PCR buffer and the DNA polymerase contained in the Advantage cDNA Polymerase kit (Clontech #8417-1).

Component (per 50 µl reaction)		**Final Concentration**
10x PCR Buffer	5 µl	10 mM
Sterile dH_2O	37 µl	NA
10mM dNTP mix	1 µl	0.2 mM
5' primer (20 µM)	1 µl	0.4 µM
3' primer (20 µM)	1 µl	0.4 µM
DNA polymerase	1 µl	
Total Volume	**46 µl**	

(5) Perform 35-40 cycles of PCR with the following conditions:
95°C x 20 sec;
60°C x 30 sec;
72°C x 30 sec.

(6) After amplification, 10 ml of the PCR products are separated by electrophoresis on 1.6% agarose gels in 0.5x Tris borate/EDTA buffer and visualized by ethidium bromide staining or GelStarR nucleic acid gel stain under UV illumination, and photographed;

b. **Fine-tuned Competitive PCR Amplification**

From the preliminary competitive PCR, determine which ten-fold dilution produces PCR MIMIC and target cDNA template bands of equal intensity. Then use the MIMIC dilution tube ten-fold less dilute to start making the two-fold serial dilutions. For example, if the M_3 dilution gives PCR MIMIC to target bands of about equal intensity, begin the two-fold serial dilution series with M_2. Briefly,

(1) Label six 0.5 ml microcentrifuge tubes $2M_1$-$2M_6$;

(2) To make two-fold serial dilution series, place 5 ml of the selected MIMIC dilution in each tube. Then,

Concentration (attomole/µl)*	Tube Label	
1.0	M_2	MIMIC dilution solution from Section 2a
5.0×10^{-1}	$2M_1$	Add 5 µl M_2, mix and change pipette tip
2.5×10^{-1}	$2M_2$	Add 5 µl $2M_1$, mix and change pipette tip
1.25×10^{-1}	$2M_3$	Add 5 µl $2M_2$, mix and change pipette tip
6.25×10^{-2}	$2M_4$	Add 5 µl $2M_3$, mix and change pipette tip
3.125×10^{-2}	$2M_5$	Add 5 µl $2M_4$, mix and change pipette tip
1.56×10^{-2}	$2M_6$	Add 5 µl $2M_5$, and mix

* attomole = 10^{-18} moles

(3) Set up six new tubes for PCR and proceed for cytokine amplification as previously described for the preliminary competitive PCR;

Add to a tube for each dilution:

2 µl	cDNA from RT
2 µl	one dilution of the MIMICs (i.e., $2M_1$ to $2M_6$)
46 µl	PCR Master Mix
50 µl	Final volume

(4) After amplification, 10 µl of the PCR products are separated by electrophoresis on 1.6% agarose gels in Tris borate/EDTA buffer and visualized by ethidium bromide staining or GelStar® nucleic acid gel stain under UV illumination, and photographed. The negative of the picture is then used for quantitative analysis as described in CALCULATIONS;

Calculations

1. Quantitative Analysis of Competitive PCR

After electrophoresis of the PCR samples, the quantitation of the amount of the target in the PCR sample is based on the determination of which two-fold serial dilution gives target and MIMIC bands of equal intensity. In our experience, visual inspection of an ethidium bromide (or GelStar®)-stained gel is sensitive enough to detect changes as low as the M7 serial dilution. The Polaroid negative from the picture is used in an image densitometric analysis using a Molecular Dynamics Densitometer. After subtraction of the background values, the density ratio of the competitor band to the target mRNA is determined and the amount of cytokine mRNA is calculated by interpolation between the known amounts of competitor which produces densities greater than and less than the target densities. Since equal aliquots of the same cDNA would be analyzed for several cytokines, the final result can be expressed as a ratio between them (e.g., IL-4/IFN-γ ratio).

One example of competitive PCR using PCR MIMIC™ is shown in Figure 5. In this example, competitive PCR for IFN-γ and IL-4 has been performed on equal aliquots of the same cDNA prepared from peripheral blood lymphocytes of one woman suffering from preeclampsia. After PCR amplification, PCR products were separated by electrophoresis on a 1.6% agarose gel and visualized by ethidium bromide staining under UV illumination and photographed. In this example, products from fine-tuned competitive PCR amplification of two-fold serial dilutions of IFN-γ and IL-4 PCR MIMIC™ and peripheral blood lymphocyte cDNA are shown (Figure 5). The presence of IFN-γ mRNA was visible as a series of bands (lower bands) of a different size in respect to the competitor ones (upper bands) (Figure 5A). In the same specimen, IL-4 mRNA was also determined (Figure 5B).

Results

1. The GAPDH amplification of a newly synthesized cDNA is very important both to make sure that the reverse transcription reaction (RT) succeeded and to check the efficiency of the cDNA synthesis. If from agarose gel electrophoresis of the PCR products no band is present corresponding to the GAPDH gene product, the RT reaction either failed or the amount of RNA used in the reaction was too small or has been degraded by ribonucleases. Thus, it is recommended to start a new RT reaction using newly-synthesized RNA. If from the agarose gel electrophoresis, the GAPDH band is present but it is not sharp and bright, the quality of the starting material was not good (i.e., ratio 260/280 nm of < 1.6) or too few RNA molecules have been utilized in the cDNA synthesis;
2. The interpretation of the results from a competitive PCR is generally easy and straightforward. However, there always exists the possibility of abnormal results. In the case of a competitive PCR using non-homologous fragments as internal controls (i.e., PCR MIMIC™), we can envision at least two sources of abnormal results:
   a. The first concerns the amount of template (cDNA) used in the PCR reaction. It is, in fact, very important to chose the right cDNA dilution before starting the preliminary competitive cytokine amplification. When the cDNA is too diluted, a weak signal is generated. This will prevent the mRNA quantification even when using the lowest serial MIMICs dilution. In this event, it is better either to start with a new, less-diluted cDNA or, if possible, to increase the volume added to the PCR reaction;

b. The second source of abnormal results concerns the possibility of a lack of signal from the specific cytokine amplification. After agarose gel electrophoresis of the PCR products, only the bands corresponding to the competitor are visible while there is no signal from the template. In the presence of a good quality cDNA (as testified by the GAPDH amplification), the lack of specific amplification might mean either that the cDNA is too diluted or that there is almost no mRNA encoding that particular cytokine tested in the sample. It might be helpful to try again to amplify the same cDNA for the specific cytokine in the absence of the competitor (i.e., MIMIC™).

Procedure Notes

1. Special precautions must be adopted when extracting RNA from a small amount of tissue or cells (< 10^6 cells or < 10mg of tissue). In these circumstances, a carrier (e.g., 5-10 mg RNase-free [Molecular Biology Grade] glycogen) for the precipitation of RNA may be added at the beginning of RNA extraction or prior to precipitating the RNA with isopropanol. This procedure will facilitate handling of the RNA and improve yields;
2. To ensure optimal RT-PCRs, all RNA preparations should be examined by denaturing agarose gel electrophoresis.[22] The integrity of RNA will be exhibited by the presence of sharp ribosomal (rRNA) bands (28S and 18S), with the 28S band about twice as intense as the 18S band;
3. In competitive PCR studies, contaminating genomic DNA can produce incorrect results because of its potential to act as a second competitor in the PCR thus affecting final mRNA quantitation. One effective method in removing genomic DNA from the total RNA preparation consists of treating the sample with DNAase I prior to RT-PCR. This procedure is sufficient to destroy all the contaminant DNA, while completely preserving the respective mRNAs.[23] For this purpose, one can use the MessageClean™ kit, GenHunter Corporation.
   a. Add the following components to a 0.2 or 0.5 ml RNAse-free tube:

Component	Amount
Total RNA	1-10 µg
10x reaction Buffer	2 µl
Dnase I (10unit/µl)	1 µl
DEPC-H_2O	to 20 µl

   b. Incubate for 30 min at 37°C;
   c. Incubate for 5 min at 75°C to heat inactivate the enzyme;
   d. Place on ice for 1 min;
   e. Collect the reaction by brief centrifugation.
   This mixture can be directly used for RT-PCR.
4. For the first strand cDNA synthesis (RT-PCR) it is preferable to use at least 1 µg of RNA for each reaction. This amount may be increased up to 5 µg per reaction in tissue samples with low mononuclear cell infiltrate or in cases of inappropriate handling or storage of the specimen. First strand cDNA synthesis reactions are routinely primed using oligo (dT). However, random hexameric primers may be used if the sequence to be detected is near the 5' end of a long mRNA.
5. A number of new constructs sharing the same features as pAW108 are commercially available and the conditions for the appropriate use described in detail by the vendor. The majority of them do not need to radiolabel the amplified material nor to run the PCR product in polyacrylamide gels. Ethidium bromide staining of bands present in agarose gels is generally sufficiently informative to determine the quantity of the original target RNA. However, particular care must be devoted to the determination of the correct range of concentrations of the competitor cRNA to be used. These may vary substantially, consistent with the variation in quantity of the target segment to be tested. Large (e.g., 10x10) dilutions can be used for a first approximation, followed by other tests in which the recognized optimal range is subdivided more precisely;
6. When assessing cytokine mRNA using non-homologous DNA competitor fragments (i.e., PCR MIMIC™), prepare PCR master mixes for the PCR reagents which are common to all tubes, such as 10x PCR buffer, dNTPs 10 mM, 5' and 3' primers, cDNA from RT and DNA polymerase. Add cDNA and DNA polymerase last. Then, start thermocycling immediately.
7. When assessing cytokine mRNA using non-homologous DNA fragments, GelStar® nucleic acid stain can be used instead of ethidium bromide to visually detect changes between target and control. This procedure is recommended when working with low competitor dilution. In this case, it would be better to utilize separate equipment for gel electrophoresis that will only be utilized for GelStar® nucleic acid gel stain to avoid interference with traces of ethidium bromide.
8. When scanning the photographic images, the negative image is preferred because it contains a wider range of densities than the positive image.
9. Clinical applications:
   The analysis of cytokine mRNA expression in peripheral blood and/or in diseased tissues of individuals affected by autoimmune, infectious or inflammatory disorders has become increasingly important. In fact, the study of the pattern of cytokine production and secretion by activated immune cells can help clarify disease pathogenesis as well as opening new strategies for therapeutic intervention (i.e., blocking antibodies against a particular cytokine). Differential activation of T-helper (TH1) and TH2-type cell subsets plays a crucial role in the resistance

or susceptibility to a variety of infectious and autoimmune diseases. For example, the immune dysregulation observed in individuals infected with human immunodeficiency virus (HIV) during progression towards AIDS could be accounted for by a shift from a TH1 to a TH2-type cytokine profile.[23] Conversely, activation of TH1-type cells has been observed in recent-onset insulin-dependent diabetic patients,[18] a common and severe organ-specific autoimmune disease. Furthermore, substantial evidence has been accumulated from animal models supporting the involvement of TH1 type cytokine in the development of other organ-specific autoimmune diseases.[24] Conversely, a protective role in autoimmunity has been suggested for the TH2 type response.[24] A type 1 response is typically characterized by increased levels of IL-2, TNF-α and IFN-γ. Those cytokines support the activation of macrophages, cytotoxic T cells and T cell-mediated delayed-type hypersensitivity. By contrast, a type 2 response is characterized by production of interleukins 4, 5, 6, and 10, which stimulate production of mast cells and eosinophils, while sustaining B-lymphocyte activation.

References

1. Oppenheim JJ, Ruscetti FW, Faltynek C: Cytokines, In: Basic and Clinical Immunology, Sites DP and Terr AI (eds), pp. 78-100, 1991.
2. Mullis KB, Faloona FA: Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. Methods Enzymol 155:335-350, 1987.
3. Larrick JW: PCR mimicscompetitive DNA fragments for use as internal standards of quantitative PCR. BioTechniques 14:244-249, 1993.
4. Weidmann E, Whiteside TL, Giorda R, Herberman RB, Trucco M: The T cell receptor Vß usage in tumor-infiltrating lymphocytes and blood of patients with hepatocellular carcinoma. Cancer Research 52:5912-5918, 1992.
5. Horikoshi T, Danenberg KD, Stadlbauer THW, Volkenandt M, Shea LCC, Aigner K, Gustavsson B, Leichman L, Frösing R, Ray M, Gibson NW, Spearks CP, Danenberg PV: Quantitation of thymidylate synthase, dihydrofolate reductase, and DT-diaphoroase gene expression in human tumors using the polymerase chain reaction. Cancer Research 52:108-116, 1992.
6. Kinoshita T, Imamura J, Nagai H, Shimotohno K: Quantification of gene expression over a wide range by the polymerase chain reaction. Anal. Biochem. 206:231-235, 1992.
7. Tam PE, Schmidt AM, Messner RP: Modified tissue pulverization technique and evaluation of dihydrofolate reductase amplification as a pan-tissue RT PCR control. PCR Methods & Applications 3:71-72, 1993.
8. Stassi G, De Maria D, Trucco G, Rudert W, Testi R, Galluzzo A, Giordano C, Trucco M: Nitric oxide primes pancreatic ?-cells for Fas-mediated destruction in IDDM. J Exp Med 186:1193, 1997.
9. Siebert PD, Larrick JW: Competitive PCR. Nature 359:557-558, 1992.
10. Celi FS, Zenilman ME, Shuldiner AR: A rapid and versatile method to synthesize internal standards for competitive PCR. Nucleic Acids Res. 21:1047, 1993.
11. Siebert PD, Larrick JW: PCR MIMICS: competitive DNA fragments for use as internal standards in quantitative PCR. BioTechniques 14:244-249, 1993.
12. Becker-André M, Hahlbrock K: Absolute mRNA quantification using the PCR: A novel approach by a PCR-aided transcript titration assay (PATTY). Nucleic Acids Res 17:9437-9446, 1989.
13. Gilliand G, Perrin S, Blanchard K, Bunn HF: Analysis of cytokines in mRNA and DNA, detection and quantitation by competitive PCR. Proc Natl Acad Sci USA 87: 2725-2729, 1990.
14. Wang A, Doyle MV, Mark DF: Quantitation of mRNA by the polymerase chain reaction. Proc Natl Acad Sci USA 86:9717-9721, 1989.
15. Platzer C, Richter G, Uberla K, Muller W, Blocker H, Diamantstein T, Blankenstein T: Analysis of cytokine mRNA levels in interleukin-4-transgenic mice by quantitative polymerase chain reaction. Eur J Immunol 22, 1179-1184, 1992.
16. Landgraf A, Reckmann B, Pingoud A: Direct analysis of polymerase chain reaction products using enzyme-linked immunosorbent assay techniques. Analytical Biochemistry 198:86-91, 1991.
17. Überla K, Pltazer C, Diamantstein T, Blankenstein T: Generation of competitor DNA fragments for quantitative PCR. PCR Methods and Applications 1:136-139, 1991.
18. Hussain MJ, Maher J, Warnock T, Vats A, Peakman M, Vergani D. Cytokine overproduction in healthy first degree relatives of patients with IDDM. Diabetologia 41:343-349, 1998.
19. Chomczynski P, Sacchi N: Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Analytic Biochemistry 162:156-159, 1987.
20. Luppi P, Rudert WA, Zanone MM, Stassi G, Trucco G, Finegold D, Boyle GJ, Del Nido P, McGowan FX, Trucco M: Idiopathic dilated cardiomyopathy: a superantigen-driven autoimmune disease. Circulation, 1998, In press.
21. Luppi P, McKnight C, Mathie M, Faas S, Rudert WA, Stewart-Akers AM, Trucco M, DeLoia JA: Evidence for Superantigen Involvement in Preeclampsia. Submitted, 1998.
22. Sambrook J, Fritsch EF, Maniatis T: Molecular Cloning: A Laboratory Manual, Second Edition. Cold Spring Harbor Laboratory Press, 1989, Vol. 1:7.43-7.45.
23. Huang Z, Fasco MJ, Kaminsky LS: Optimization of DNase I removal of contaminating DNA from RNA for use in quantitative RNA-PCR. BioTechniques 20:1012-1020, 1996.
23. Clerici M, Shearer GM: The Th1-Th2 hypothesis of HIV infection: new insights. T-cell function 15:575-581, 1994.24.Liblau RS, Singer SM, McDevitt HO: Th1 and Th2 CD4+ T cells in the pathogenesis of organ-specific autoimmune diseases. Immunol Today 16:34-38,1995.

Table I. Properties of various cytokines.

Cytokine	MW	Principal CellSources	Primary Type of Activity	Preeminent Effects
IL-1	17,500	Macrophages and others	Immunoaugmentation	Inflammatory and hematopoietic
IL-2	15,500	TH1 cells, some CTL	T and B growth factor	Activates T and NK cells
IL-3	14,000-28,000	TH1, TH2, some CTL	Hematopoietic growth factor	Promotes growth of early myeloid progenitor cells
IL-4	20,000	TH2 cells	T and B cell growth factor; promotes IgE reactions	Promotes IgE switch and eosinophilia
IL-5	18,000	TH2 cells	Stimulates B cells and eosinophils	Growth factor for B cells and polyclonal immunoglobulin production
IL-6	22,000-30,000	Fibroblasts and TH2	Hybridoma growth factor; augments inflammation	Generates pre-B and pre-T cells and polyclonal immunoglobulin production
IL-7	25,000	Stromal cells	Lymphoprotein	Generates pre-B and pre-T cells and is lymphocyte growth factor
IL-8	8,800	Macrophages and others	Chemoattracts neutrophils and T lymphocytes	Regulates lymphocyte homing and neutrophil infiltration
G-CSF	18,000-22,000	Monocytes and others	Myeloid growth factor	Generates neutrophils
M-CSF	18,000-26,000	Monocytes and others	Macrophage growth factor	Generates macrophages
GM-CSF	14,000-38,000	TH1, some TH2, some CTL	B-cell differentiation, inhibits T cell growth, activates macrophages	Myelopoiesis
IFN-α IFN-β IFN-γ	18,000-20,000 25,000 20,000-25,000	Leukocytes Fibroblasts TH_1 cells and NK cells	Antiviral, antiproliferative and immunomodulating	Stimulates macrophages and NK cells; Induce cells membrane antigens (e.g., MHC)
TNF-α TNF-β	17,000 18,000	Macrophages and T lymphocytes (TH1 and TH2)	Inflammatory, immunoenhancing and tumoricidal	Vascular thromboses and tumor necrosis
TGF-γ	25,000	Platelets, T cells and macrophages	Fibroplasia and immunosuppression	Wound healing and bone remodeling, activates neutrophils

Table II. Sequences of 5' primers and 3' primers of 12-target genes.

			Size of PCR Product (bp)	
MRNA	**5' Primers**	**3' Primers**	**mRNA**	**cRNA**
TNF	5'-CAgAgggAAgAgTTCCCCAg-3'	5'-CCTTggTCTggTAggAgACg-3'	325	301
M-CFS	5'-gAACAgTTgAAAgATCCAgTg-3'	5'-TCggACgCAggCCTTgTCATg-3'	171	302
PDGF-A	5'-CCTgCCCATTCggAggAAgAg-3'	5'-TTggCCACCTTgACgCTgCg-3'	225	301
PDGF-B	5'-gAAggAgCCTgggTTCCCTg-3'	5'-TTTCTCACCTggACAggTCT-3'	217	300
APO-E	5'-TTCCTggCAggATgCCAggC-3'	5'-ggTCAgTTgTTCCTCCAgTTC-3'	270	301
LDL-R	5'-CAATgTCTCACCAAgCTCTg-3'	5'-TCTgTCTCgAggggTAgCTg-3'	258	301
HMG	5'-TACCATgTCAggggTACgTC-3'	5'-CAAgCCTAgAgACATAATCATC-3'	246	303
IL-1α	5'-gTCTCTgAATCAgAAATCCTTCTATC-3'	5'-CATgTCAAATTTCACTgCTTCATCC-3'	420	308
IL-1β	5'-AAACAgATgAAgTgCTCCTTCCAgg-3'	5'-TggAgAACACCACTTgTTgCTCCA-3'	388	306
IL-2	5'-gAATggAATTAATAATTACAAgAATCCC-3'	5'-TgTTCAgATCCCTTTAgTTCCAg-3'	222	305
PDGF-R	5'-TgACCACCCAgCCATCCTTC-3'	5'-gAggAggTgTTgACTTCATTC-3'	228	300
LPL	5'-gAgATTTCTCTgTATggCACC-3'	5'-CTgCAAATgAgACACTTTCTC-3'	277	300

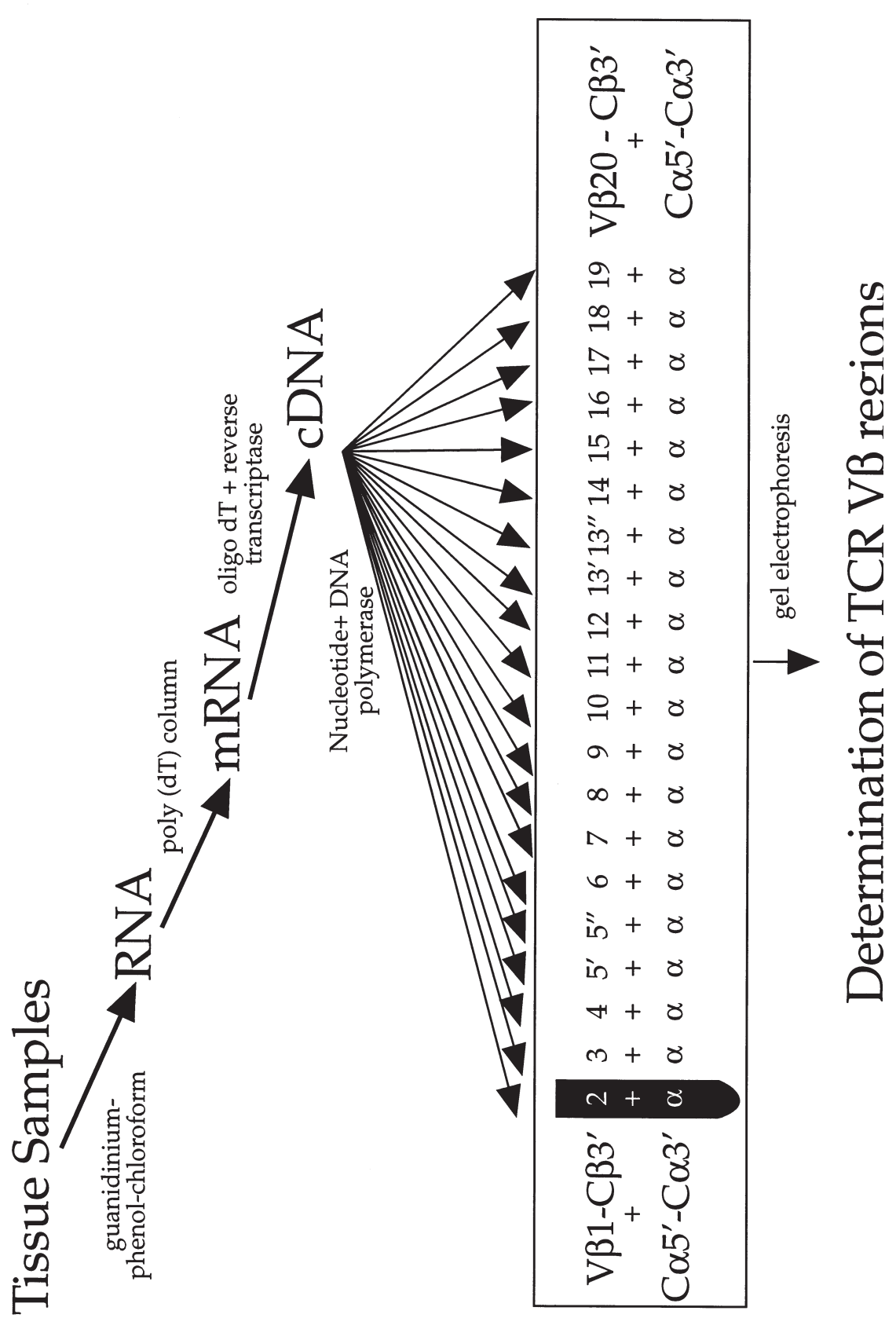

Tissue Samples
guanidinium-phenol-chloroform
RNA
poly (dT) column
mRNA
oligo dT + reverse transcriptase
cDNA
Nucleotide+ DNA polymerase
CR
Vβ1-Cβ3′ + Cα5′-Cα3′
2 3 4 5′ 5″ 6 7 8 9 10 11 12 13′ 13″ 14 15 16 17 18 19
+ + + + + + + + + + + + + + + + + + +
α α α α α α α α α α α α α α α α α α α
Vβ20 - Cβ3′ + Cα5′-Cα3′
gel electrophoresis
Determination of TCR Vß regions

Figure 1. Detection of T cell receptor (TCR) variable region gene expression in T cells.

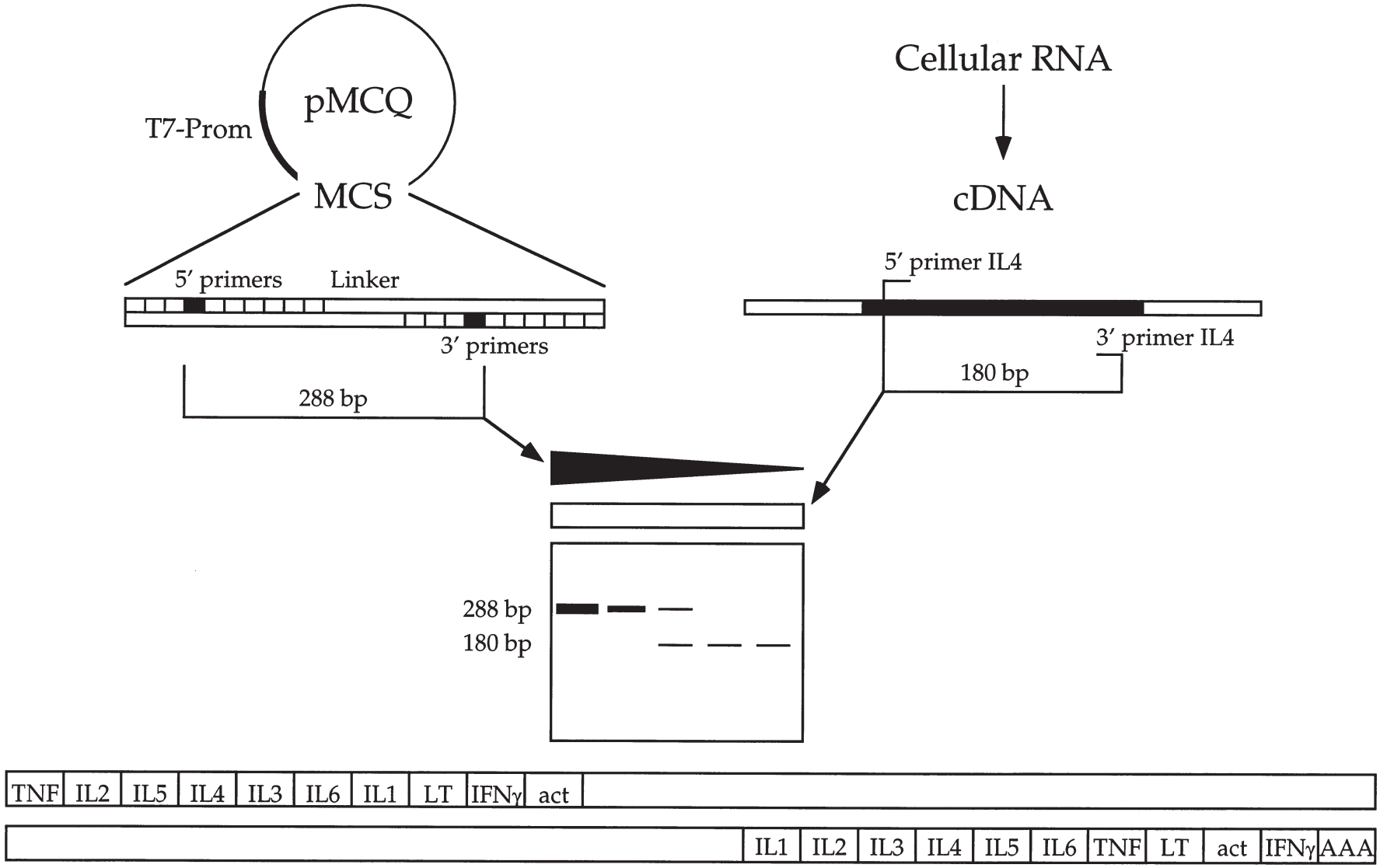


Figure 2. Experimental outline for cytokine mRNA quantification by competitive PCR. The plasmid pMCQ contains primers specific for mouse cytokine quantification. This method determines the amount of target cDNA by comparing the intensity of the bands of its amplified material with those of serially diluted control fragments. The order of the 5' and 3' primer specific sequences in plasmid pMCQ is shown. MCS = multiple cloning site. (Modified from reference 15).

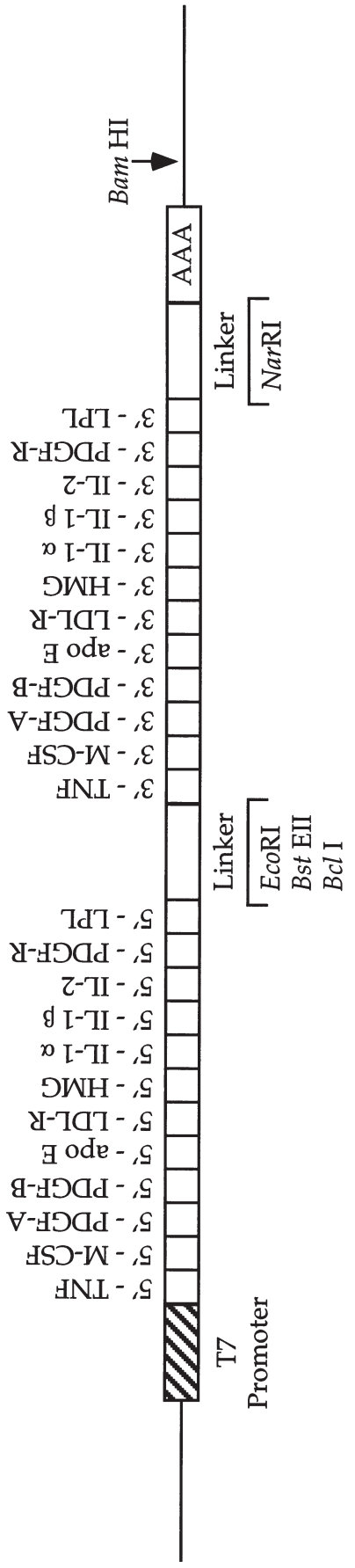


Figure 3. Structure of pAW108. The plasmid contains 5' primers of 12 human cytokine target genes connected in sequence followed by the complementary sequences of the 3' primers in the same order. Restriction enzyme linkers are placed after the set of 5' primers and after the set of 3' primers. The multiple primer region is flanked upstream by the T7 polymerase promoter and downstream by polyadenylated sequences. The corresponding oligonucleotides of 5' primers and 3' primers are listed in Table 2 (Modified from reference 14).

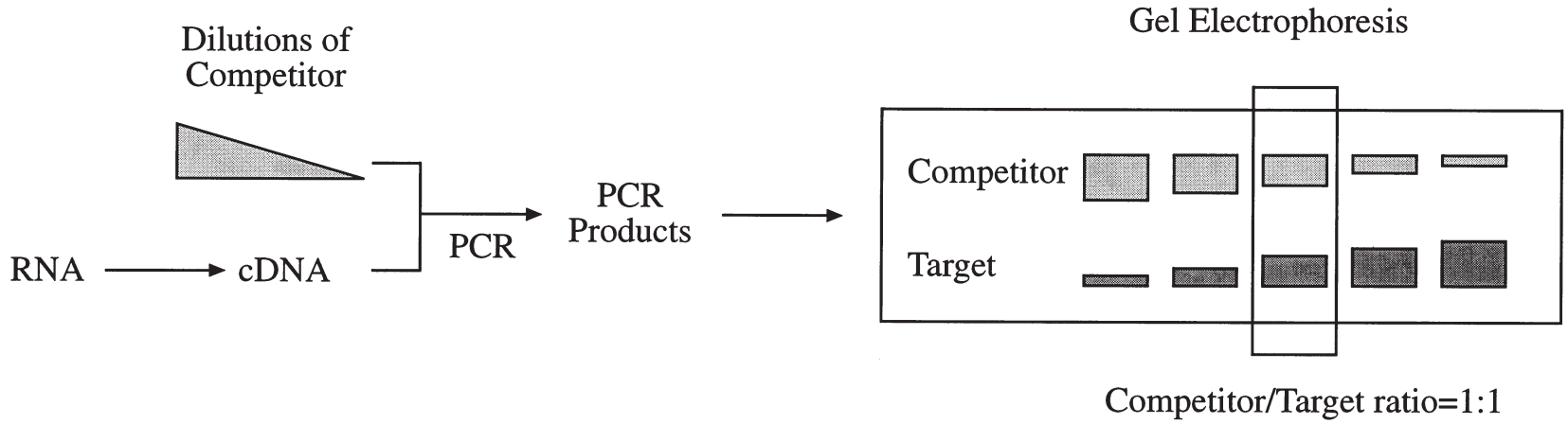
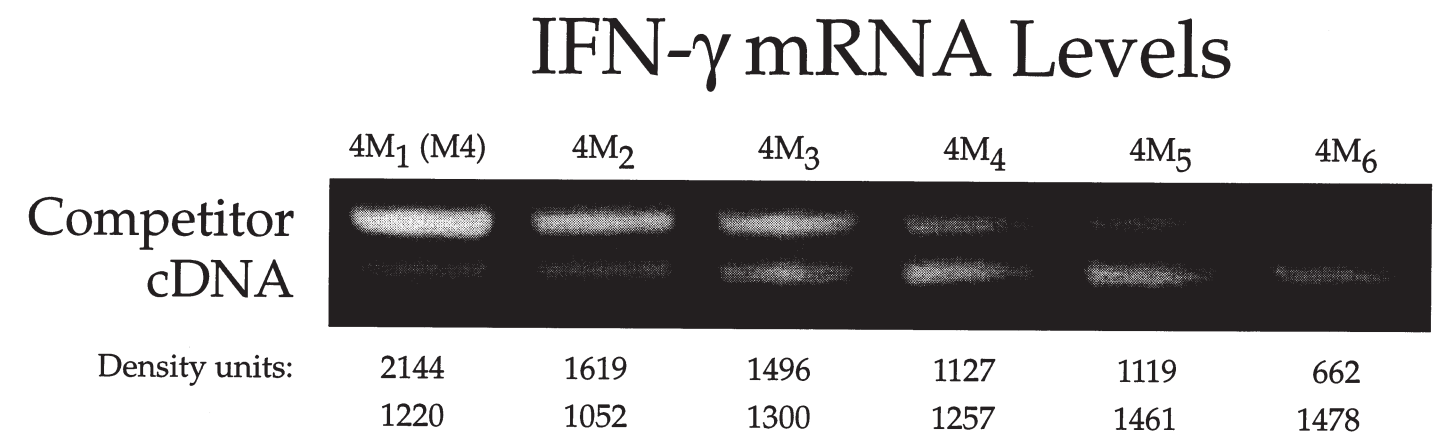


Figure 4. Schematic diagram of competitive PCR utilizing a competitor DNA fragment differing in size from the target sequence. A dilution series of the competitor is added to a constant amount of cDNA. Following amplification, samples of the PCR products are resolved by gel electrophoresis, and the ratios of the amplified competitor and target products are quantified. The amount of cytokine mRNA is calculated by interpolation between the known amounts of competitor which produce densities nearest to the target densities.

A



B

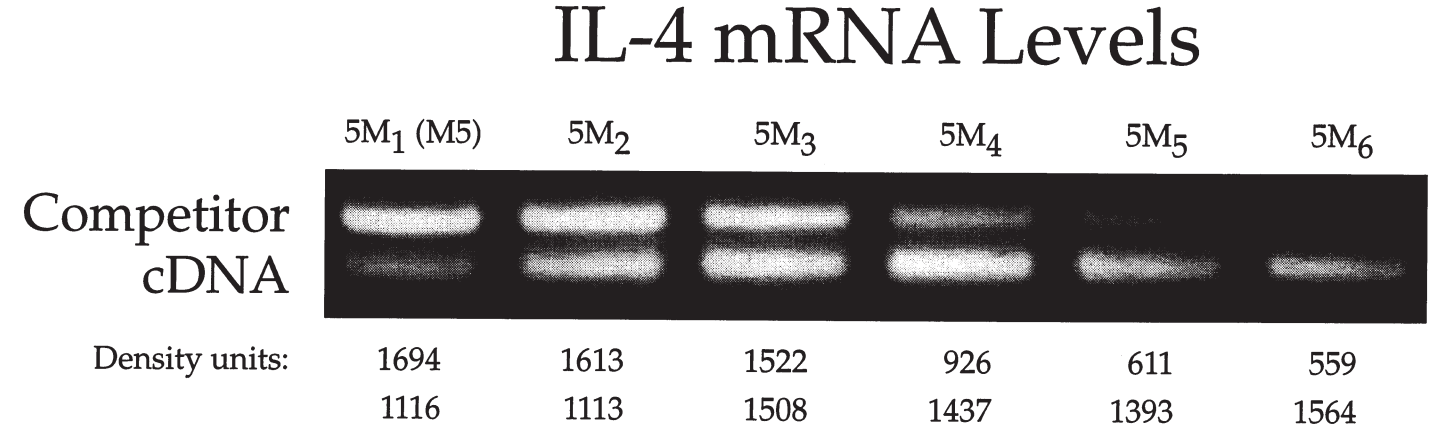


Figure 5. Competitive PCR analysis for IFN-γ and IL-4. Equal aliquots of cDNA prepared from peripheral blood lymphocytes of a patient suffering from preeclampsia were amplified in the presence of serial dilutions of a specific competitor cDNA for either IFN-γ (A) or IL-4 (B) as described in the relative section (PROCEDURE: B2). PCR products were separated by electrophoresis on a 1.6% agarose gel and visualized by ethidium bromide staining under UV illumination and photographed. The upper band is due to amplification of the competitor cDNA and the lower band is due to amplification of the target cDNA. The particular two-fold competitive dilution is shown above each pair of bands and the densitometric measurements are shown beneath the bands. In the first lane of IFN-α (A) and IL-4 amplification (B) the amount of competitor was 15,000 molecules and 1,500 molecules, respectively.

MHC Microsatellite Analysis

Maureen P. Martin and Mary Carrington

Principle and Purpose

Microsatellites are repeated tandem sequences of DNA, most of which are $(CA)_n$ repeats. In the human genome they are interspersed about every 30-60 kilobases (kb) in euchromatic regions of DNA. Their function is largely unknown but they may play a role in recombination and gene regulation. They are useful genetic markers because of their high degree of polymorphism and Mendelian inheritance, and have been used in linkage mapping, construction of evolutionary trees, forensic medicine, and relatedness testing. In addition they are easily typed and require relatively small amounts of starting material. More than 70 microsatellites within and flanking the human major histocompatibility complex (MHC) have been identified. Some of these have been used in a number of studies involving the MHC, including the mapping of the hemochromatosis gene, and the identification of recombinant chromosomes.

Genes of the human MHC have been shown to be associated with a variety of diseases including insulin-dependent diabetes mellitus (IDDM), narcolepsy, ankylosing spondylitis, celiac disease, Behçet's disease, psoriasis vulgaris, multiple sclerosis, idiopathic nephrotic syndrome, inflammatory bowel disease, asthma, and autosomal dominant cerebellar ataxia to name a few. The strong linkage disequilibrium between loci in the HLA class I, II and III regions complicates the ability to identify a specific locus involved in disease resistance and/or susceptibility. Data from our laboratory suggest that typing microsatellites within the MHC may allow for accurate mapping of disease associated regions. In five diseases studied (IDDM, multiple sclerosis, narcolepsy, uveitis, and C2 deficiency), MHC microsatellites confirmed the previously known regions of disease associations. Due to linkage disequilibrium between these markers and closely linked genes, they may also aid in identifying new areas of involvement in disease in addition to the known disease regions.

Identity at HLA-A, -B, and –DRB1 loci is a good indication of genomic identity across the entire MHC in related individuals, but these do not necessarily define the major genomic transplantation loci in all cases. Several studies indicate that differences at DP may be more critical than previously thought, and minor histocompatibility antigens may play a role in GVHD and graft rejection. In addition, it is possible that there are as yet unidentified MHC loci telomeric to HLA-A which may also be important in transplantation. Microsatellite typing may provide a more accurate and thorough means for determining identity across the HLA complex including those regions outside the classical HLA loci. We have developed a semi-automated fluorescence-based typing technique for typing microsatellites within and around the MHC, which is highly efficient, accurate, and reproducible. Figure 1 shows the location of the eighteen microsatellites described herein, relative to the class I, II, and III regions. This method can also be applied to other microsatellites not listed, including those neighboring minor histocompatibility antigens.

Specimen

Genomic DNA from any source such as whole blood, peripheral blood lymphocytes, serum, or plasma can be used. Any standard protocol for DNA extraction should be suitable. DNA should be placed in a -20^0C freezer for long-term storage, or 4^0C for short-term storage.

Reagents and Supplies

A. PCR

1. Oligonucleotide primers (10mM) complementary to the 5′ and 3′ sequences flanking the microsatellites (see Table 1). These can be obtained commercially from a number of sources (e.g., Gibco BRL, Gaithersburg, MD). The 5′ primers are fluorescently tagged at the 5′ end with 6-FAM, HEX, or TET phosphoramidites. The fluorescent-labeled primers can be obtained from ABI and should be stored in the dark at –20°C.
2. Sterile deionized distilled water.
3. 10X PCR buffer (100mM Tris-HCL pH 8.3, 500mM KCl, 0.01% w/v gelatin). This buffer is available commercially (Perkin-Elmer, Foster City, CA), but may also be made in the lab. Stored at 4^0C.
4. Deoxynucleotide stock solution (2mM each dATP, dTTP, dGTP, and dCTP) available from USB, Cleveland, OH. Stored at -20^0C.
5. 25mM $MgCl_2$. Stored at 4^0C.
6. *Taq* DNA polymerase, preferably Ampli*taq* gold™ from Perkin-Elmer. This enzyme confers a "hot start" to the polymerase chain reaction that greatly improves the efficiency and specificity of the multiplex PCR. Stored at -20^0C.

B. Electrophoresis

1. Long-Ranger™ sequencing gel solution (50% stock solution) available from FMC bioproducts, Rockland, ME. Store at room temperature and protect from light. Alternatively, a 40% stock solution of acrylamide and bis-acrylamide (19:1) stored at room temperature and protected from light, can be used. Avoid inhalation and contact with skin since these chemicals are neurotoxins. Gloves, goggles and work in a fume hood *must* be worn when handling these solutions.
2. Molecular biology grade crystalline urea. Avoid inhalation and contact with skin, eyes and clothing, as urea is a chemical hazard.
3. Ammonium Persulfate (APS). Stored in an airtight container in the dark and kept dry.
4. Water-free TEMED (N,N,N′,N′-tetramethylethylenediamine) stored in a tightly sealed container. TEMED is a chemical and fire hazard. Work under a hood and wear gloves to avoid inhalation and contact with skin, eyes and clothing.
5. 10X Tris-borate-EDTA (TBE) stock solution (0.89M Tris, 0.89M boric acid, 0.02M disodium EDTA, pH 8.3) and is commercially available. If the laboratory chooses to prepare its own stock solution, ultra-pure reagents must be used and the buffer must be filter sterilized (0.2mm filter).
6. Loading buffer (5:1 deionized Formamide: 25mM EDTA with 50 mg/ml Blue Dextran) and should be made up fresh each time a gel is run.
7. Fluorescent-labeled molecular mass marker (Genescan-350 Tamra); available from ABI and stored at 4^0C.
8. Mixed bed, ion-exchange resin – AG 501-X8 resin from BIO RAD, Hercules, CA.
9. Alconox™ (New York, NY) detergent.

Instrumentation and Equipment

1. Thermal cycler, preferably Perkin Elmer 9600 or 9700, or similar machine with fast ramp times. PCR can be performed in other thermal cyclers but it may be necessary to adjust the PCR conditions.
2. Thin-walled 0.2 ml PCR tubes in strips of 8 or 12, and strip caps or rubber plate covers (available from Perkin-Elmer).
3. ABI model 377 DNA sequencer.
4. Gel cassette (ABI). This is useful for gel pouring and transportation of the plates, and eliminates the need for using clips to prevent leakage while the gel is being poured.
5. Glass plates (36 cm) from ABI.
6. Gel spacers from ABI (0.2 mm).
7. Fifty well comb from ABI.
8. Nalgene (Rochester, NY) 115 ml 0.2 mm cellulose nitrate filter unit.
9. Gel injection device from ABI.
10. 60 cc syringe.
11. Heating block or oven.

Calibration

The Genescan analysis software which is used for data analysis, is designed for ease of use, flexibility and automation. It automatically sizes DNA fragments, allowing more accurate and faster analysis than other methods such as radioactive labeling. Once the samples are loaded, there is no need to manipulate data or manually enter analyzed data, so the possibility of human error is reduced. The accompanying manual is extremely easy to follow and assistance by telephone is readily available.

1. Use fluorescent amidite standards to create the matrix, which will be used to analyze the gels. Refer to the Genescan analysis software user's manual for instructions on creating a matrix file. Although the dyes used to label the PCR products fluoresce at different wavelengths, there is some overlap in the spectra. Matrix files are mathematical matrices that correct for this overlap. Application of the matrix file to the pre-analyzed fragment data will correct for spectral overlap, and ensures that the spectral data collected from gel to gel is consistent.
2. The Genescan program has default analysis parameters but the user can also set them up. The parameters include analysis range, peak detection settings, size range of peaks to be tabulated, and size calling method. Refer to the Genescan user's manual for details.
3. A fluorescence labeled size standard made up of DNA fragments of known sizes (35-350 base pairs) is run in each lane of the gel. This results in accurate and precise molecular length determination of fragments of unknown size because the size standard and the unknown fragments undergo exactly the same forces. The Genescan software will compensate for band-shift artifacts due to variations in the gel and/or the run (see the Genescan analysis manual).

Quality Control

It is quite feasible to run all or some samples twice because of the low cost per sample and the small amount of DNA needed for the assay. This would allow confirmation of typings for each sample. Known controls such as the 10th International Workshop B lymphoblastoid cell lines (HTCs) can be run on each gel in order to verify size calling and check for accuracy from gel to gel. Verification of the values calculated by the Genescan software for the size standard can be achieved by viewing the electropherograms generated by each sample. This permits verification that the peaks were properly detected and that the size standard was properly matched (see the Genescan manual for details).

Procedure

A. PCR

1. Amplification is performed in two separate multiplex reactions for each sample in a total volume of 20 µl for the following two groups of microsatellites:
   a. Group 1: D6S273, D6S291, TAP1CA, RING3CA, D6S276, G51152.
   b. Group 2: MOGCA, D6S265, MIB.
   Amplify all other microsatellites in individual reactions, unless multiplex reactions are developed for the additional markers.
2. Make the following primer mixes (per sample) for groups 1 and 2:

Group 1:	D6S273.5	0.60 µl
	D6S273.3	0.60 µl
	D6S291.5	0.45 µl
	D6S291.3	0.45 µl
	TAP1CA.5	0.25 µl
	TAP1CA.3	0.25 µl
	RING3CA.5	0.60 µl
	RING3CA.3	0.60 µl
	D6S276.5	0.90 µl
	D6S276.3	0.90 µl
	G51152.5	0.25 µl
	G51152.3	0.25 µl
	TOTAL	**6.10 µl**
Group 2:	MOGCA.5	0.80 µl
	MOGCA.3	0.80 µl
	D6S265.5	0.30 µl
	D6S265.3	0.30 µl
	MIB.5	0.35 µl
	MIB.3	0.35 µl
	TOTAL	**2.90 µl**

3. Make up the following PCR cocktails (per sample):

Group 1:	Primer mix	6.1 µl
	10X PCR buffer	2.0 µl
	2mM dNTP mix	2.0 µl
	25mM $MgCl_2$	0.4 µl
	Taq polymerase	0.1 µl
	dDW	7.4 µl
	DNA (20-50 ng/µl)	2.0 µl
	TOTAL	**20.0 µl**
Group 2:	Primer mix	2.9 µl
	10X PCR buffer	2.0 µl
	2mM dNTP mix	2.0 µl
	25mM $MgCl_2$	0.4 µl
	Taq Polymerase	0.1 µl
	DDW	10.6 µl
	DNA	2.0 µl
	TOTAL	**20.0 µl**

All other loci:	5′ primer	0.8 µl
	3′ primer	0.8 µl
	10X PCR buffer	2.0 µl
	2mM dNTP mix	2.0 µl
	25mM $MgCl_2$	0.4 µl
	Taq polymerase	0.1 µl
	DDW	11.9 µl
	DNA	2.0 µl
	TOTAL	**20.0 µl**

4. Perform PCR using the following conditions for the Perkin Elmer 9600/9700 and Amplitaq Gold™ *Taq* polymerase. It is possible to use other PCR machines and or enzymes but the conditions may need to be altered to achieve successful amplification.

   Groups I and II, HLAC-CA1, HLA BC-CA2, MICA, D6S105:

	94^0C	8 min	
Denature	94^0C	15 sec	┐
Anneal	55^0C	15 sec	┘ X 30 cycles
Extend	72^0C	30 sec	
	72^0C	30 min	

   D6S439 – as above, but with an annealing temperature of 57^0C.

   DRA CA1, BAT2 CA – as above, but with an annealing temperature of 60^0C.

   DD6S510 – as above, but with an annealing temperature of 65^0C.

DQCARII:	94^0C	8 min	
	94^0C	30 sec	┐
	65^0C	30 sec	┘ X 5 cycles
	72^0C	1 min	
	72^0C	30 min	
	94^0C	30 sec	┐
	57^0C	30 sec	┘ X 15 cycles
	72^0C	30 sec	
	94^0C	30 sec	┐
	55^0C	1 min	┘ X 5 cycles
	72^0C	2 min	
	72^0C	30 min	

Preparation of PCR Product for Electrophoresis

5. Dilute and pool amplified samples into 2 groups as follows:

   PANEL A

Group I	1:10	5.0 µl
Group I	1:25	2.0 µl
dDW		43.0 µl
TOTAL		**50.0 µl**

   PANEL B

D6S510	1:50	2.0 µl
HLAC-CA1	1:40	5.0 µl
HLA BC-CA2	1:20	5.0 µl
D6S105	1:10	10.0 µl
BAT2 CA	1:40	2.5 µl
DRA CA1	1:30	3.3 µl
D6S439	1:20	5.0 µl
MICA	1:30	3.3 µl
DQCARII	1:10	10.0 µl
dDW		53.9 µl
TOTAL		**100.0 µl**

6. Combine 2.5 µl of pooled, diluted PCR product from each panel with 3.5 µl standard/loading buffer (2.5 µl formamide + 0.5 µl blue dextran (50 mg/ml in 25mM EDTA) + 1 µl Genescan-350 Tamra).
7. Denature samples at 96^0C for 2 min, and place on ice.

Denaturing Gel Electrophoresis

8. Prepare the gel mix as follows:
9. Weigh out 36 g molecular biology grade urea in a 250 ml beaker.
10. Add 54 ml dDW and 10 ml 50% Long Ranger gel solution.
11. Add 5 g AG 501-X8 resin.
12. Add a stir bar, cover with parafilm and stir on stir plate until all the urea crystals have dissolved (approximately 30 min).
13. While the gel solution is being stirred, assemble the gel apparatus after cleaning the plates with Alconox™ (see ABI PRISM™ 377 manual).
14. Filter the gel solution in a Nalgene 115 ml 0.2 µm cellulose nitrate filter unit for 5 min in order to degas the solution (5 min from the time all of the gel solution has passed through the filter), and transfer to a 100 ml graduated cylinder.
15. Add 10 ml 10X TBE and adjust the volume with dDW to 80 ml (if necessary).
16. Add 500 µl freshly made 10% ammonium persulfate and 50 µl TEMED. Swirl to mix but be careful not to introduce air bubbles.
17. Draw the gel solution up into a 60 ml syringe, clear any air bubbles from the syringe and screw the syringe into the coupler on the gel pouring apparatus.
18. With a slow steady action dispense the gel solution until it fills all the space between the plates. Allow some excess solution to pool at the top.
19. Insert the comb, and place two clips over the comb equidistant from the center.
20. Remove the syringe and gel pouring apparatus, and allow the gel to polymerise for at least two hours.
21. Assemble the gel on the ABI PRISMTM 377 sequencer (see manual for details), and load the upper and lower buffer chambers with 1X TBE running buffer. You will need approximately 1400 ml of 1X buffer.
22. Load 2 µl of each sample mix.
23. Run the gel as per the instructions in the ABI 377 manual, for 2 hours.

Calculations

Collect and analyse the data using the ABI Prism Genescan 2.1 analysis software and the Genotyper 2.0 DNA fragment analysis software. These software allow interpretation of nucleic acid fragment size and quantitation data by converting it into user defined results which can be transferred to a database for storage and analysis (see the Genescan analysis software user's manual). The third order least squares size calling option is used to calculate the size calibration curve and the 35-350 base pair fragments of the Genescan-350 ladder are used for the calibration curve.

Results

Allele designation is based on the size of the product (number of base pairs). The color of each microsatellite and the approximate size range of the alleles are given below. The size range of the products and the fluorescent label are chosen so that several loci (in this case 9 loci are included in each panel) with alleles in the same size range can be run in a single lane, each labeled with a different color (see below). The Genescan analysis software automatically analyses the data and also allows the user to confirm and fine-tune the analysis. In addition, the data can be displayed in a number of ways including electropherograms, tabular data, or a combination of both.

Microsatellite Locus	Allele size range (bp)	Fluorescent Label*
	PANEL A	
D6S276	63-151	Yellow
G51152	193-251	Yellow
MOGCA	122-160	Green
D6S265	176-218	Green
MIB	257-295	Green
D6S273	139-163	Blue
TAP1CA	187-211	Blue
RING3CA	221-243	Blue
D6S291	166-186	Blue
	PANEL B	
D6S105	144-164	Yellow
MICA	180-200	Yellow
D6S510	170-200	Green
BAT2CA	135-155	Green
D6S439	270-300	Green
HLAC-CA1	100-120	Green
HLABC-CA2	90-130	Blue
DRACA1	240-270	Blue
DQCARII	180-230	Blue

*Yellow = HEX, Green = TET, Blue = 6-FAM

Procedure Notes

1. Fluorescently labeled primers should be stored at –20^{0}C protected from light to prolong their shelf life. Aliquoting the primers into smaller volumes will cut down on repeated freeze-thawing.
2. Ampliq*taq* Gold™ (Perkin-Elmer Corp., Norwalk, CT), which is a modified *Taq* polymerase is useful in improving the efficiency and specificity of the PCR. The enzyme does not become enzymatically active until it is exposed to a high temperature soak (95^{0}C for 8-12 min). This essentially confers a hot start to the PCR.
3. *Taq* polymerase can cause non-templated addition of a nucleotide (usually adenosine) to the 3′ end of the amplicon, which presents a potential source of error in genotyping. In order to increase the likelihood that adenosine will be uniformly added to the amplified products, the final extension step (72^{0}C) is lengthened to 30 min. This is important because alleles are assigned based on the number of bases. It is therefore possible that for a given microsatellite an allele may be identified as either the modified or unmodified product.
4. Allele peaks seen outside the expected size range may be due to bleed-through from other colors because of off-scale data. Electrophoresis should be repeated using less sample. Primers that are not fully optimized may also result in a similar problem.
5. With allele peaks of high intensity, the Genescan software may call many small peaks. One reason for this is that too much PCR product is loaded resulting in a high background level. Repeat electrophoresis using less sample.
6. Pour gels carefully and gently to avoid the formation of bubbles which can distort the sample path and affect lane tracking.
7. Be certain that the outer surface of the gel plates, particularly the region where the laser reads the gel (the lower end), are clear of all dust particles, lint, water spots and acrylamide before assembling on the sequencer.
8. Alconox is used to wash the plates because it does not leave a residue which may result in background fluorescence.

Limitations of Procedure

Occasionally there are small discrepancies in the sizing of alleles as determined by direct sequencing versus fluorescence-based typing, which may be due to a number of possibilities. In some cases adenosine may have been added to the amplified product. Alternatively, the primary and secondary structure of the DNA fragment may affect its mobility and cause it to run slower or faster than predicted. However, the results obtained by Genescan analysis are reproducible from gel to gel such that if they differ from the sequence by one or two base pairs, they do so consistently for all samples.

References:

1. Beck S, Abdulla S, Alderton RP, Glynne RJ, Gut IG, Hosking LK, Jackson A, Kelly A, Newell WR, Sanseau P, Radley E, Thorpe KL, Trowsdale J, Evolutionary dynamics of non-coding sequences within the class II region of the MHC. J. Mol. Biol. 255:1-13, 1996.
2. Beck S, Alderton R, Kelly A, Khurshid F, Radley E, Trowsdale J, DNA sequence analysis of 66 kb of the human MHC class II region encoding a cluster of genes for antigen processing. J. Mol. Biol. 228: 433-441, 1992.
3. Bouissou C, Pontarotti P, Crouau-Roy B, A precise meiotic map in the class I region of the human major histocompatibility complex. Genomics 30: 486-492, 1995.
4. Bowcock AM, Ruiz-Linares, A, Tomfohrde J, Minch E, Kidd JR, Cavalli-Sforza LL, High resolution of human evolutionary trees with polymorphic microsatellites. Nature 368: 455-457, 1994.
5. Carrington M, Marti D, Wade J, Klitz W, Barcellos L, Thomson G, Chen J, Truedsson L, Sturfelt G, Alper C, Awdeh Z, Huttley G, Microsatellite markers in complex disease: Mapping disease-associated regions within the human major histocompatibility complex. In: Microsatellites: Evolution and Applications, Goldstein DB and Schlötterer C, eds., Oxford University Press, Oxford, England; 1998. (In Press).
6. Carrington M, Wade J, Selection of transplant donors based on MHC microsatellite data – Correction to previously published material. Hum. Immunol. 51: 106-109, 1996.
7. Carrington M, Dean M, A polymorphic dinucleotide repeat in the third intron of TAP1. Hum. Mol. Genet. 3: 218, 1994.
8. Feder JN, Gnirke A, Thomas W, Tsuchihashi Z, Ruddy DA, Basava A, Dormishian F, Domingo Jr R, Ellis MC, Fullan A, Hinton LM, Jones NL, Kimmel BE, Kronmal GS, Lauer P, Lee VK, Loeb DB, Mapa FA, McClelland E, Meyer NC, Mintier GA, Moeller N, Moore T, Morikang E, Prass CE, Quintana L, Starnes SM, Schatzman RC, Brunke KJ, Drayna DT, Risch NJ, Bacon BR, Wolff RK, A novel MHC class I-like gene is mutated in patients with hereditary haemochromatosis. Nat. Genet. 13: 399-408, 1996.
9. Foissac A, Crouau-Roy B, Fauré S, Thomsen M, Cambon-Thomsen A, Microsatellites in the HLA region: an overview. Tissue Antigens 49: 197-214, 1997.
10. Gallagher G, Eskdale J, Miller S, A highly polymorphic microsatellite marker in the human MHC class III region, close to the BAT2 gene. Immunogenetics 46: 357-358, 1997.
11. Grimaldi MC, Clayton J, Pontarotti P, Cambon-Thomsen A, Crouau-Roy B, A new highly polymorphic microsatellite marker in linkage disequilibrium with HLA-B. Hum. Immunol. 51: 89-94, 1996.
12. Gyapay G, Morisette J, Vignal A, Dib C, Fizames C, Millaseau P, Marc S, Bernardi G, Lathrop M, Weissenbach J, The 1993-94 Généthon human genetic linkage map. Nat. Genet. 7: 246-339, 1994.
13. Hagelberg E, Gray IC, Jeffreys AJ, Identification of the skeletal remains of a murder victim by DNA analysis. Nature 352: 427-429, 1991.
14. Hamada H, Kakunaga T, Potential Z-DNA forming sequences are highly dispersed in the human genome. Nature 298: 396-398, 1982.

15. Lin L, Jin L, Kimura A, Mignot E, DQ microsatellite association studies in three ethnic groups. Tissue Antigens 50: 507-520, 1997.
16. Martin MP, Harding A, Chadwick R, Kronick M, Cullen M, Lin L, Mignot E, Carrington M, Characterization of 12 microsatellite loci of the human MHC in a panel of reference cell lines. Immunogenetics 47: 131-138, 1998.
17. Martin M, Mann D, Carrington M, Recombination rates across the HLA complex: use of microsatellites as a rapid screen for recombinant chromosomes. Hum. Mol. Genet. 4: 423-428, 1995.
18. Mizuki N, Ota M, Kimura M, Ohno S, Ando H, Katsuyama Y, Yamazaki M, Watanabe K, Goto K, Nakamura S, Bahram S, Inoko H, Triplet repeat polymorphism in the transmembrane region of the MICA gene: A strong association of six GCT repititions with Behçet disease. Proc. Natl. Acad. Sci. USA 94: 1298-1303, 1997.
19. Roth M-P, Dolbois L, Borot N, Amadou C, Clanet M, Pontarotti P, Coppin H, Three highly polymorphic microsatellites at the human myelin oligodendrocyte glycoprotein locus, 100 kb telomeric to HLA-F. Hum. Immunol. 43: 276-282, 1995.
20. Smith JR, Carpten JD, Brownstein MJ, Ghosh S, Magnuson VL, Gilbert DA, Trent JM, Collins FS, Approach to genotyping errors caused by nontemplated nucleotide addition by Taq DNA polymerase. Genome Res. 5: 312-317, 1995.
21. Tamiya G, Ota M, Katsuyama Y, Shiina T, Oka A, Makino S, Kimura M, Inoko H, Twenty-six new polymorphic microsatellite markers around the HLA-B, -C and -E loci in the human MHC class I region. Tissue Antigens 51: 337-346, 1998.
22. Weber JL, Kwitek AE, May PE, Zoghbi HY, Dinucleotide repeat polymorphism at the D6S105 locus. Nucleic Acids Res. 19: 968, 1991.
23. Weissenbach J, Gyapay G, Dib C, Vignal A, Morisette J, Millaseau P, Vaysseix G, Lathrop M, A second-generation linkage map of the human genome. Nature 359: 794-801, 1992.
24. Yang SY, Milford E, Hammerling V, Dupont B, Description of the reference panel of B-lymphoblastoid cell lines for factors of the HLA system: the B-cell line panel designed for the Tenth International Histocompatibility Workshop. In: Immunobiology of HLA (vol. 1), B Dupont, ed., Springer, New York; p.11, 1989.

Table 1 Primers used for microsatellite typing

Locus	5′ Primer Sequence	26. 3′ Primer Sequence
D6S276	5-tcaatcaaatcatccccagaag	5-ccttctttgcagactgtcacc
D6S105	5-gggattacaggcaggagccac	5-gaaggagaattgtaattccg
MOGCA	5-gaaatgtgagaataaaggaga	5-gataaaggggaactactaca
D6S510	5-aatgggctactacttcacacc	5-caacacactgatttccatagc
D6S265	5-agtcaccctactgtgctatc	5-atcgaggtaaacagcagaaag
HLAC-CA1	5-tagactagctcttgactact	5-gcctgccaccataccccact
HLABC-CA2	5-tgggcaatgagtcctatgac	5-tgccatttggccctaaatgc
MIB	5-gcttcacccgatcagtagaagac	5-gcatggtgtcagagatagtcaggtc
D6S273	5-ggagaagttgagtatttctgc	5-accaaacttcaaattttcgg
MICA	5-ccttttttcagggaaagtgc	5-ccttaccatctccagaaactgc
BAT2CA	5-ctccagcctggataacag	5-acaagggctttaggaggtct
DRACA1	5-tggaatctcatcaaggtcag	5-acatttgtatgcttcagatg
DQCARII	5-gcactatcattaaatttgctttccacagtac	5-gattcataaggcaagaatccagcatattgg
G51152	5-ggtaaaattcctgactggcc	5-gacagctcttcttaacctgc
TAP1CA	5-gctttgatctcccccctc	5-ggacaatattttgctcctgagg
RING3CA	5-tgcttatagggagactaccg	5-gaggtaatgtcacaggatggg
D6S439	5-gatgatttaagtttcctgtggacc	5-ttcaaggacagcctcaggg
D6S291	5-ggcattcaggcatgcctggc	5-ggggatgacgaattattcactaact

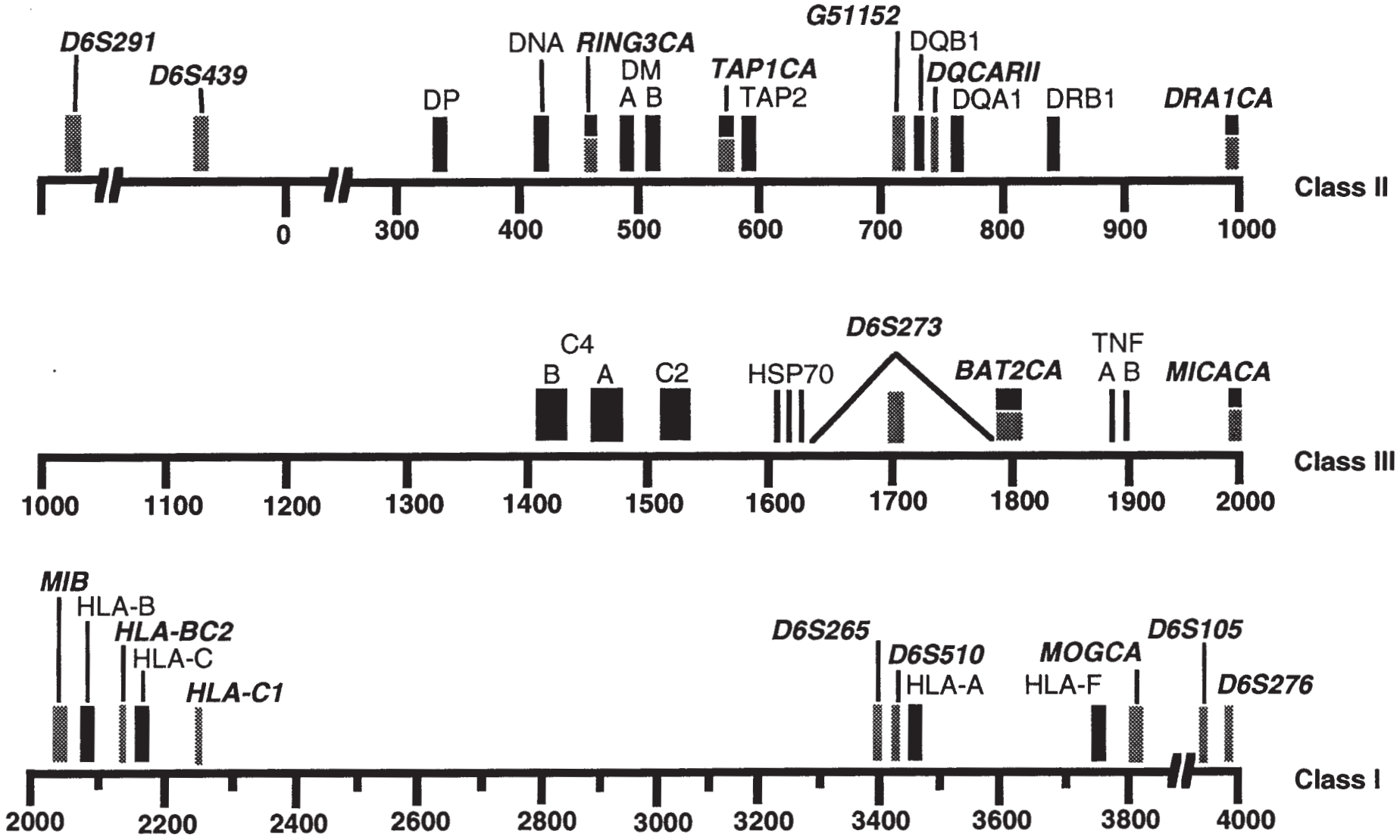

D6S291
D6S439
DP
DNA
RING3CA
DM
A B
TAP1CA
TAP2
G51152
DQB1
DQCARII
DQA1
DRB1
DRA1CA
Class II
0
300
400
500
600
700
800
900
1000
C4
B
A
C2
HSP70
D6S273
BAT2CA
TNF
A B
MICACA
Class III
1000
1100
1200
1300
1400
1500
1600
1700
1800
1900
2000
MIB
HLA-B
HLA-BC2
HLA-C
HLA-C1
D6S265
D6S510
HLA-A
MOGCA
HLA-F
D6S105
D6S276
Class I
2000
2200
2400
2600
2800
3000
3200
3400
3600
3800
4000

Figure 1

Basic Principles and Quality Assurance of Immunofluorescence and Flow Cytometry

Mary S. Leffell and Robert A. Bray

Purpose

The combined techniques of immunofluorescence and flow cytometry provide a powerful tool for both basic research and clinical application. Neither technique is new. Immunofluorescence originated in the 1940's when Coons first coupled the fluorescent dye, anthracene, to antibody molecules. Today's hi-tech flow cytometers evolved from cell and particle counters which measured objects by changes in electrical resistance or light refraction. With the advent of monoclonal antibodies to a vast array of cellular markers and with the development of reasonably priced cytometers which utilize small air cooled lasers and state-of-the-art computers, the possibilities for cytometric analysis in cell biology have become seemingly unlimited. Specifically in the area of histocompatibility, there are an increasing number of applications for flow cytometry, including flow crossmatches and immunological monitoring. These specific topics will be discussed in later chapters. This brief discussion is intended to serve only as an introduction to the basic principles of immunofluorescence and flow cytometry. The reader is directed to the reference section of this chapter for more detailed references related to flow cytometry and immunofluorescence.

Immunofluorescence

In the technique of immunofluorescence an antibody molecule provides specificity by binding to the desired target antigen. The antigen-antibody interaction is made visible by use of a fluorescent dye, or fluorochrome, which is either coupled directly to the antibody molecule or coupled to a secondary antibody molecule which has specificity for the primary antibody. Fluorochromes are molecules which, when exposed to radiation (usually in the ultraviolet range), become excited. This excitation is transient and when the molecules return to the ground state, the absorbed energy is released (emitted) as radiation of a characteristic and longer wavelength within the visible range (Figure 1). Many molecules are capable of fluorescence and certain cellular components, including fibers and granules, can exhibit weak auto-fluorescence. One of the problems with anthracene, the first dye tried by Coons, was that its characteristic bluish-green fluorescence was difficult to distinguish from weak cellular auto-fluorescence. In the 1950's, Coons and his colleagues first described the conjugation of fluorescein isothiocyanate (FITC) to antibody molecules. Fluorescein with its characteristic yellow-green fluorescence (emission at 520nm) is easily distinguished from auto-fluorescence and has remained one of the most widely used fluorochromes.

Table 1 lists some commonly used fluorochromes with their respective maximum excitation wavelengths and their major characteristic emission wavelength. It is useful to know these properties when choosing fluorochromes for use in two or three color cytometric analysis, which will be discussed in the next section. To conjugate a fluorochrome to an antibody, it must first be converted to a derivative with a chemically active group (e.g., isothiocyanate), which is then used to couple the dye to the protein antibody molecule. The molar ratio of fluorochrome : antibody is important, as labeling ratios of < 1.0 result in weak fluorescence. Conversely, higher labeling ratios (>3.0) give the antibody molecules an increased net negative charge which can increase non-specific staining. High molar ratios of fluorochrome : antibody may also affect antibody binding. The choice of antibody to use is dependent primarily upon the availability of a reagent of the desired specificity and suitable binding affinity. The labeling efficiency does vary with the species, isotype, and/or subtype of immunoglobulin being used. For example, rabbit immunoglobulins cannot be labeled with more than 2-3 fluorochrome molecules without losing antigen binding capability, while sheep or goat immunoglobulins can withstand heavier coupling (up to 5-7 fluorochrome molecules/immunoglobulin molecule). For this reason, it is still often useful to know the labeling ratio of these reagents in use to understand problems with staining efficiency or to mix reagents for two color analysis. Today there are many excellent commercially produced monoclonal antibodies available (Table 2), and the conjugation of fluorochromes to these antibodies is generally performed by the manufacturer. The categorizing of monoclonal antibodies (MoAb) is based upon the distinct cellular molecule (most commonly a glycoprotein) with which they react and, in turn, are clustered based on their reactivity. Hence, the terminology *"Cluster Defined"* or *"Cluster Designation"* commonly abbreviated, *"CD"*. The term CD is generally followed by a number (e.g., CD3) and is used to identify all antibodies that react with a given cellular molecule. For example, Leu-4 (Becton-Dickinson, Inc), OKT3 (Ortho

Pharmaceuticals), T3 (Coulter, Inc.) all recognize the CD3 molecule which is expressed on the surface and in the cytoplasm of mature T lymphocytes. These antibodies may recognize distinct or similar epitopes on the molecule. For example, cells stained with OKT3 may also be stained with Leu-4 (albeit at a lower intensity) since Leu-4 recognizes a distinct epitope on CD3 which is only partially blocked by the presence of OKT3.

The technique of immunofluorescence has many applications in clinical and research laboratories aside from its use as a specific probe in flow cytometry. Fluorescent labeled antibodies have been used extensively for the detection and localization of antigens, such as infectious agents, in cells and tissues; for the determination of antibodies to infectious agents in patient sera; and for studying the localization of antibodies, complement, or immune complexes in autoimmune disorders. Currently, immunofluorescence is used in histocompatibility laboratories primarily for the identification of cell surface markers (Immunophenotyping) or the detection of alloantibodies directed against HLA antigens. Detection of cell surface antigens or alloantibodies requires staining of live cells in suspension with appropriate fluorescent labeled antibodies. Staining is performed by either a direct approach or a two-step indirect technique (Figure 2).

In the direct staining technique, the specific antibody used is directly conjugated with a fluorochrome. This antibody is allowed to react and bind directly with living cells in suspension. The cells to be tested are first washed with a balanced salt solution containing protein, usually 1% bovine serum albumin, to remove adsorbed serum proteins that may interfere with staining. The labeled antibody is then allowed to react with the cells for 20-30 minutes to permit stabilization of antibody-antigen binding. Many cell surface antigens are capable of "capping"; i.e., when bound by a specific antibody, the antigens coalesce to one pole of the cell and are endocytosed. Since capping is an energy dependent process, it can be prevented by staining at a low temperature (4°C) and/or by the addition of an energy inhibitor such as sodium azide (NaN_3) to the staining solution. After the staining incubation, cells are again washed with media or balanced Salt/protein solution to remove unbound antibody. The stained cells then be observed under a fluorescent microscope or subjected to flow cytometric analysis.

The indirect technique of fluorescent staining was developed by Mellors et al. in the 1950's. In this two-step or "sandwich" approach, the primary antibody is not labeled but is allowed to react and bind to the cells under study. After a binding incubation and the usual washes, the second antibody, a fluorochrome labeled antiglobulin, is added. Use of the antiglobulin greatly increases the sensitivity of immunofluorescence and allows detection of membrane antigens present in low concentration or in sparse distribution. The indirect technique is also used for enhanced sensitivity crossmatches by using an anti-human immunoglobulin to detect patient alloantibodies. Unfortunately, the indirect technique is subject to more non-specific, background staining. This non-specific staining is often due to the propensity for antiglobulins to bind to receptors for the Fc portion of immunoglobulins (FcR) found on many cells. Because most FcR react primarily with aggregated immunoglobulins or with immune complexes, non-specific staining due to FcR can be reduced by prior ultracentrifugation of antiglobulin reagents or by the use of an $F(ab)'_2$, fragments as the secondary antibodies.

Flow Cytometry

In the simplest terms, flow cytometry is a process whereby multiple characteristics of individual cells or particles are simultaneously analyzed. The key word in this definition is "individual", because one of the most important components of a cytometer is the sample-handling or fluidics system which sends cells through the cytometer's flow cell in single file. Each cell then passes through a focused light source and sensors record the interactions of the cell or particle with the light source. Two other features which add to the power and sensitivity of cytometers are: 1) a refined light source (most commonly a laser) and 2) sophisticated computer systems for data analysis. Combining these features makes multiparameter analysis of both structural and functional properties of cells and subpopulations possible. Structurally, cytometric analysis can measure cell size, complexity (i.e.; shape and cytoplasmic granularity), pigment content, DNA/RNA content, and even chromatin structure. Some of the functional properties which can be studied include: redox state, membrane integrity, membrane permeability and fluidity, surface charge, surface receptors, cytoplasmic Ca^{++} content, DNA synthesis, and intracellular pH. As was mentioned above, cytometers are being used in histocompatibility labs primarily for the identification of cell surface markers and detection of alloantibodies reactive with cell HLA molecules. This brief discussion will, therefore, be limited to the principles of cytometric analysis of surface markers on lymphoid cells, but it should be remembered that cytometry can be used for many other purposes.

Figure 3 schematically illustrates the major components of a flow cytometer. A prepared cell suspension (which usually consists of cells stained with a fluorochrome conjugated antibodies) is injected from a pressurized container into a sheath stream of buffer. Injection of the cell suspension into this outer sheath fluid flowing in the same direction hydrodynamically focuses the cells to the center of the sample stream. The cells are then aligned in single file and pass through the flow cell and into the path of a focused beam of light. When each cell intersects the light beam, both the scattered light from the beam and the emitted fluorescence will be collected and analyzed. Fluorescence emission is, of course, dependent upon the cytometer's light source being of the appropriate wavelength for fluorescent excitation and the cells being labeled with appropriate fluorochrome conjugated antibodies. Sensors or photomultiplier tubes (PMTS) are placed to pick up the light signals generated by the passage of each cell through the light beam. Electronic signals are then transferred to the instrument s computer for analysis. For virtually all elected events ("gated" data) or total events (List Mode) may be stored for subsequent analysis.

Obviously the light source is a critical component of a cytometer. Until recently most flow cytometers were equipped with high powered, laser plasma tubes which required dedicated power supplies, cooling system, and special environmental precautions for controlling temperature and light. Such instruments are costly to purchase and maintain and, in addition, require highly trained and experienced operators. The most common light source on these instruments is a large, 5 watt argon laser which produces a strong blue-green 488nm beam suitable for exciting many popular fluorochromes,

such as fluorescein and phycoerythrin (Table 1). Although these larger instruments are still available today, they are almost exclusively used in research settings. For routine clinical testing, there are smaller (and less expensive) cytometers powered by air cooled lasers that are much more compatible with clinical laboratory needs in terms of their initial cast, space and support requirements. The air cooled argon lasers that are currently avialable emit much less energy (15 to 25mw at 488nm excitation) than their older, more powerful counterparts. However, due to significant improvements in the optical systems these smaller instruments are actually providing better sensitivity than their larger predecessors. Other small air cooled lasers such as helium-neon (633nm excitation) or helium-cadmium (325nm excitation) may be additionally employed, allowing the possibility of multi-beam instruments and several color analyses.

Regardless of the light source, the most appealing feature of any cytometer is its capability to perform simultaneous multiparameter analysis of individual cells. In Figure 3, sensors are shown for four parameters: forward angle light scatter (FALS or FSC); 90° light scatter (orthogonal right angle light scatter [RALS]), side scatter (SSC); orange and green fluorescence. For simplicity, these parameters will be used for a discussion of how cell populations may be analyzed, but it should be remembered that other parameters might also be evaluated with additional hardware. As cells pass through the focused beam of light, some light photons may be absorbed while others are refracted or scattered. Laser light that is scattered in the forward direction of the light beam is proportional to cell size. Laser light that is refracted 90° to the laser beam correlates with cell complexity. If cells are tagged with fluorochromes, the absorbed light is emitted by the fluorochrome at a longer wavelength and is detected by the fluorescence detectors that are 90° to the sample stream. If the cells are suspended in an isotonic electrolyte solution, cell volume may also be estimated by changes in electrical resistance. Depending upon the type of cytometer, measurements of light scatter and/or volume are used to differentiate whole blood or buffy coat leukocytes into subpopulations of lymphocytes, monocytes, and granulocytes (Figure 4). Boundaries or "gates" may then be set electronically to define a window around one or more of these populations, allowing the analysis of only one cell type, from a mixed cell population. Labeling of cells with a fluorescent antibody permits the delineation of a subset within the gated population. For example, lymphocytes can be stained with a FITC conjugated monoclonal antibody to the CD3 complex, which marks mature T lymphocytes. After preliminary gating by FALS and 90° light scatter to define the total lymphocyte population, T lymphocytes can be detected by their green fluorescence.

At this point, a word or two is necessary to explain how cytometers process the light signals generated by cells interacting with the laser beam. Photons of refracted light or fluorescent emissions are converted by photomultiplier tubes into electric current with the amplitude of the current pulse correlating with the intensity of the light signal. Individual pulses are recorded on an electronic scale divided into voltage increments or channels. In addition to converting light photons into electric current, the photomultiplier tubes amplify the signal output. This signal amplification can be linear or logarithmic. With linear amplification, the current output is directly proportional to the fluorescence emission. With logarithmic amplification, a percentage increase in fluorescence intensity corresponds to a constant increase in channel number. Logarithmic amplification is generally used to study subsets of cells varying widely in fluorescence intensity in order to display all populations on the same scale. The frequency of events falling within given channels defines populations or sub-populations of cells. The frequency of events (in this case, number of lymphocytes) is plotted on the Y-axis and the relative fluorescence intensity is displayed on the X-axis. Figure 5 illustrates an example of a single parameter histogram. The curve reflects the distribution of cells among the specified fluorescent channels. The height of the curve is directly proportional to the number of cells within a given channel. Flow cytometry data may be displayed as a histogram, scatter or dot plots, or as contour plots.

Figure 6 shows an example of a leukocyte suspension analyzed for total T lymphocytes using a FITC conjugated CD3 monoclonal antibody. The total leukocyte suspension was first "gated" by FALS and 90° light scatter around lymphocytes (R1 from Figure 4) so that only the fluorescence emissions from lymphocytes are being displayed. In this example the fluorescence intensity is measured using a log scale. Two peaks of fluorescence are typically observed. The first peak, of low intensity, represents auto-fluorescence and non-specific background staining of the CD3 negative cells. The second peak, of significantly higher intensity, represents the staining pattern of the CD3 positive T lymphocytes. Analysis of the second peak allows determination of the percentage of CD3 positive lymphocytes within the total lymphocyte population analyzed. In this example, 55% of the patient's lymphocytes bear the CD3 antigen. Fluorescence intensity is often designated by a channel number and many instruments can define the mean, median, and peak channel of fluorescence intensity for a cell population. The mean or median channel of fluorescence can be used as a qualitative measure of antigen density. Increased antigen density or the increased expression of cell surface antigens will result in increased fluorescence intensity that will be indicated by a shift to higher channel values. The mean or median channel of fluorescence is also used in some flow cytometric crossmatch techniques as the criterion for determining a positive reaction.

Two, three or four (or more) color fluorescent analysis can be used to further differentiate subpopulations of cells. Multi-color analysis depends upon the use of fluorochromes with sufficiently different emission spectra such that their signals can easily be separated. Currently, a combination of FITC and phycoerythrin (PE) is the most widely used for two color analysis, because, as shown in Table 1, they each have distinct emissions of 517nm (green) and 578nm (orange) respectively, and yet they both can be excited by a single light source of 488nm. The utility of two-color analysis can be appreciated by the example of HLA-DR expression on peripheral blood lymphocytes from a bone marrow transplant recipient. MHC Class II (HLA-DR) molecules are not expressed at detectable levels on resting T lymphocytes from normal individuals, whereas they are constitutively expressed on B lymphocytes. However, after activation, HLA-DR molecules can readily be detected on T lymphocytes. If a population of lymphocytes is stained with an antibody to a monomorphic HLA-DR epitope, cytometric analysis can determine the proportion of HLA-DR positive cells, but with no differentiation between B and T lymphocytes. Use of a second antibody, for example against the T lymphocyte marker CD5, permits this discrimination. However, the CD5 antigen, which is a normal T cell antigen, may also be expressed on a subset of B cells

($CD5^+$ B cell). Figure 7 illustrates an example of two-color staining of peripheral blood lymphocytes obtained from a bone marrow transplant recipient approximately 3 months post transplant. This example of two-color flow cytometric analysis illustrates the great potential of multiparameter analysis. In this example, a PE (orange) conjugated monoclonal to CD5 is used to define all T cells and a PerCP™ (red) monoclonal to HLA-DR is used to assess the HLA-DR expression. Such analysis requires that, first, the cytometer is gated on total lymphocytes and, secondly, that appropriate controls are used to define the fluorescent intensities of both positively and negatively stained cell populations. These controls are used to establish the boundaries of four quadrants in a two parameter histogram of orange fluorescence (Fluorescence 2, X-axis) versus red fluorescence (Fluorescence 3, Y-axis). In a properly controlled analysis, cells exhibiting only background staining for orange and/or red fluorescence are displayed in Quadrant 3, while cells positive for either orange or red are shown in Quadrants 1 and 4, respectively. The doubly stained cells are displayed in Quadrant 2. In our example, four distinct fluorescent positive cell populations are illustrated. HLA-DR positive B lymphocytes (red fluorescence only) are in Quadrant 1, while non-activated T cells (orange fluorescence only) are in Quadrant 4. However, in Quadrant 2 we see two distinct clusters of cells, **R2** and **R3**. R3 represents those T cells that co-express HLA-DR, while R2 represents a subset of B lymphocytes that co-express the CD5 antigen. This unique subset of B cells is routinely observed in patients following bone marrow transplant or bone marrow reductive chemotherapy. Note, however, that the expression of the CD5 antigen is of low density compared to the expression of CD5 on the DR negative cells and that the expression of HLA-DR on T cells is of low density compared to HLA-DR expression on mature B lymphocytes.

While FITC and PE are currently in wide use for two-color fluorescent analysis, three or four color combinations offer many new potential applications. Presently, several manufacturers offer directly conjugated antibodies bearing fluorochromes which are excited at 488 nm and have emission maxima > 650 nm (Table 1). This spectral property allows these reagents to be used in combination with FITC and PE to perform 3-color flow cytometry using a single laser instrument. Three-color flow cytometry is now considered "standard practice" for the flow cytometric crossmatch. More recently, dual-laser bench-top clinical cytometers can now perform 4-color analysis. However, some multi-color analysis is, not surprisingly, complicated and presently beyond the scope of most clinical laboratories. For example, use of rhodamine, Texas Red or Allophycocyanin in combination with FITC or PE requires instruments equipped with two lasers because of the difference in the excitation wavelengths of these fluorochromes (see Table 1). While smaller instruments equipped with multiple air cooled lasers are being developed, at present multi-beam instruments are the larger and more complex cytometers more suited to research laboratories. Precise alignment and calibration of the lasers and optics in such analysis is critical and is often a nightmare for even experienced users.

One other aspect of cytometers which should be briefly mentioned is the capability of some instruments to “sort” or separate cell populations. Cell sorting has, in the past, been confined to the larger instruments and is not a function routinely needed in clinical laboratories. Sorting relies on the fluid system of the cytometer, which directs the cells into a stream, flowing single file through the flow cell. This stream can be sonicated and broken into droplets. If the flow rate is controlled, a single cell can be contained in one drop. By programming appropriate sort signals, such as green or red fluorescence, certain drops can be selected for sorting as they pass through the light source. To sort, the cell stream is momentarily deflected in an electrical field to allow the desired droplet to be collected in a reservoir , according to their net electrical charge (See Figure 3). Although sorting can result in highly purified cell populations, it is a time consuming procedure and not suited for the of large numbers of cells.

Before leaving this discussion of the combined use of immunofluorescent probes and flow cytometry, it must be stressed that, as in any good program of clinical laboratory practice, appropriate quality assurance is essential. In fact, without the proper controls, correct interpretation of cytometric data is impossible. On any cytometer, alignment of the light source and optical system must be verified routinely. Slight deviations in the alignment of the light beam with the cell stream will adversely affect the collection of FALS and 90° light scatter as well as fluorescent excitation. It is, therefore, accepted practice to daily verify the cytometer’s performance by analyzing a standard cell or particle suspension. Fluorescent latex beads or fixed, stained cells may be used for this purpose. Appropriate positive and negative controls must also be used for fluorescent antibody staining. When using monoclonal antibodies, it is vital that these controls be of the same isotype as each monoclonal being used. For applications using indirect staining techniques, such as flow cytometric crossmatches, and for two-color fluorescent analysis, isotype negative controls are especially critical. As was mentioned above, indirect staining often results in a high level of non-specific background fluorescence. While this may be due in part to FcR binding, dead or dying cells (such as often present in leukocyte suspensions being used for antibody crossmatches) will also non-specifically absorb fluorescent conjugated antiglobulins. Without a control for background staining, interpretation of a flow crossmatch by any criteria is impossible. For two color analysis, positive and negative controls are required, not only to establish the correct quadrants for definition of single and double-labeled cell populations, but also to electronically compensate for “bleed-over” of fluorescent emissions into another channel which may occur with some fluorochrome combinations. To many novices in cytometry, the necessary controls often seem as numerous as the actual specimens to be analyzed; however, it takes only a little experience to realize their necessity. Given all that can be learned from properly controlled analysis, the effort becomes worthwhile. An excellent reference that deals directly with the quality control and quality assurance of flow cytometry is **NCCLS document H42-A**, *“Clinical Applications of Flow Cytometry: Quality Assurance and Immunophenotyping of Lymphocytes”*, Approved Guideline (1998). This document is available from the NCCLS (National Committee for Clinical Laboratory Standards) by writing, calling or via the internet. (See below).

NCCLS
771 East Lancaster Ave.
Villanova, PA 19085
www.nccls.org
(215) 525-2435

References

1. Bauer KD, Duque RE, Shankey TV, eds. *Clinical Flow Cytometry: Principles and Applications*. 1993. Williams, and Wilkins Inc., pub. 634 pp.
2. Coligan JE, Kruisbeek AM, Marguiles DH, Shevack EM, Strober W. eds, The CD System of Leukocyte Surface Molecules. In: *Current Protocols in Immunology, Vol.2*. Wiley and Sons, New York. pp. A.4.1 – A.4.20.1991.
3. Colvin RB, Preffer Fl, New technologies in cell analysis by flow cytometry. Arch. Pathol. Lab. Med. 111:628-632, 1987.
4. Coon JS and Weinstein RS, eds.: *Techniques in Diagnostic Pathology, No. 2. Diagnostic Flow Cytometry*. 1991. Williams & Wilkins, Inc., pub.
5. Darzynkiewicz Z, Robinson JP, and Crissman HA. *Methods in Cell Biology: Flow Cytometry*, 2ed, Part A. Vol.#41. Academic Press, NY. 1994.
6. Darzynkiewicz Z, Robinson JP, and Crissman HA. *Methods in Cell Biology: Flow Cytometry*, 2ed, Part B. Vol.#42. Academic Press, NY. 1994.
7. Forni L, Reagents for immunofluorescence and their use for studying lymphoid cell products. In: *Immunological Methods*; I Lefovits, B Pemis, eds. Academic Press, New York; pp. 151-167, 1979.
8. Given AL: *Flow Cytometry: First Principles*. Wiley-Liss, New York, 1992. 202 pp.
9. Jackson AL and Warner NL, Preparation, staining, and analysis by flow cytometry of peripheral blood leukocytes. In: *Manual of Clinical Immunology*, 3rd ed.; NR Rose and H Friedman, eds. American Society for Microbiology, Washington, DC; pp. 226-235,1986.
10. Landay AL, Ault KA, Bauer KD and Rabiniovitch PS, eds. *Clinical Flow Cytometry*. Ann. N. Y. Acad. Sci. 1993. Vol. 677. 468 pp.
11. Leffell MS, Specialty Assays. Characterization of cell surface antigens. In: *SEOPF Tissue Typing Reference Manual*; JM MacQueen, ed.; Southeastern Organ Procurement Foundation, Richmond, VA; pp.9-7 to 9-15, 1987.
12. Lovett EJ, Schnitzer B, Keren DF, Flint A, Hudson JL, and McClatchey KD, Application of flow cytometry to diagnostic pathology. Lab. Invest. 50:115-139, 1984.
13. Segal DK, Titus JA, Stephany DA, Fluorescence flow cytometry in the study of lymphoid cell receptors. *Methods in Enzymology* 150:478-492, 1987.
14. Shapiro HM. *Practical Flow Cytometry*. Alan R. Liss, Inc., New York, 1985.

Table 1

Excitation / Emission Spectra for some commonly used fluorochromes.

Fluorochrome	Excitation Maximum (nm)	Emission Maximum (nm)
Fluorescein isothiocyanate (FITC)	490	520 (green)
Phycoerythrin-R (R-PE)	480	578 (orange)
Peridinin Chlorophyll Protein (PerCP( )	470	677 (red)
PE-APC Tandem Conjugates	480	660 (red)
Rhodamine isothiocyanate (RITC)	580	610 (red)
Allophycocyanine	650	660 (red)

Table 2

Glossary of some commonly used Cluster Defined (CD) Antgens

Cluster #	Distribution	Specificity
CD2	T Cells, NK Cells	LFA-2, CD58 (LFA-3) receptor
CD3	Mature T Cells	T cell receptor complex
CD4	T Helper Cells, Thymocytes, Monocytes, Macrophages	55kd protein that serves in MHC Class II recognition and as a receptor for HIV
CD5	Mature T Cells, Thymocytes, Subset of B Cells	67kd glycoprotein, Ligand for CD72
CD8	T cytotoxic/suppressor cells NK Cells	32-34kd glycoprotein Class I MHC recognition
CD10	B and T precursors, granulocytes CALLA (Common Acute Lymphoblastic Leukemia Antgen)	Neutral endopeptidase
CD11	11a – Pan-leukocyte 11b – Myeloid Cells, NK Cells and T suppressor cells	LFA-1; Integrin family MAC-1; Complement receptor III
CD14	Mature monocytes	53-55kd glycoprotein
CD16	Natural Killer (NK) Cells, Neutrophils and Macrophages	FcγRIII, Fc receptor for IgG
CD19	Pan B Cell	95kd glycoprotein
CD20	Mature B Cells	33-37kd glycoprotein
CD21	Mature B Cells, some T-ALLs	Complement receptor II, EBV receptor
CD25	Activated T, B and Monocytes	IL-2 Receptor, Tac
CD28	T Cells and Plasma Cells Increased following activation	Ig supergene family Ligand for B7 (CD80)
CD45	All Hematopoietic Cells except Mature Erythrocytes	Leukocyte Common Antigen, LCA
CD45RA	Most T, B and NK Cells	CD45 isoform
CD45RO	Activated T Cells	CD45 isoform; Binds CD22
CD34	Immature Hematopoietic Cells	105-120kd glycoprotein
CD56	NK Cells, Non-MHC restricted CTLs	175-185-Kd glycoprotein, N-CAM
CD80	B7.1, BBl; activated T/B cells, monocytes	Co-stimulatory molecule, binds CD28
CD86	B7.2	Co-stimulatory molecule, binds CD28
CD122	IL-2Rβ	Interleukin-2 receptor

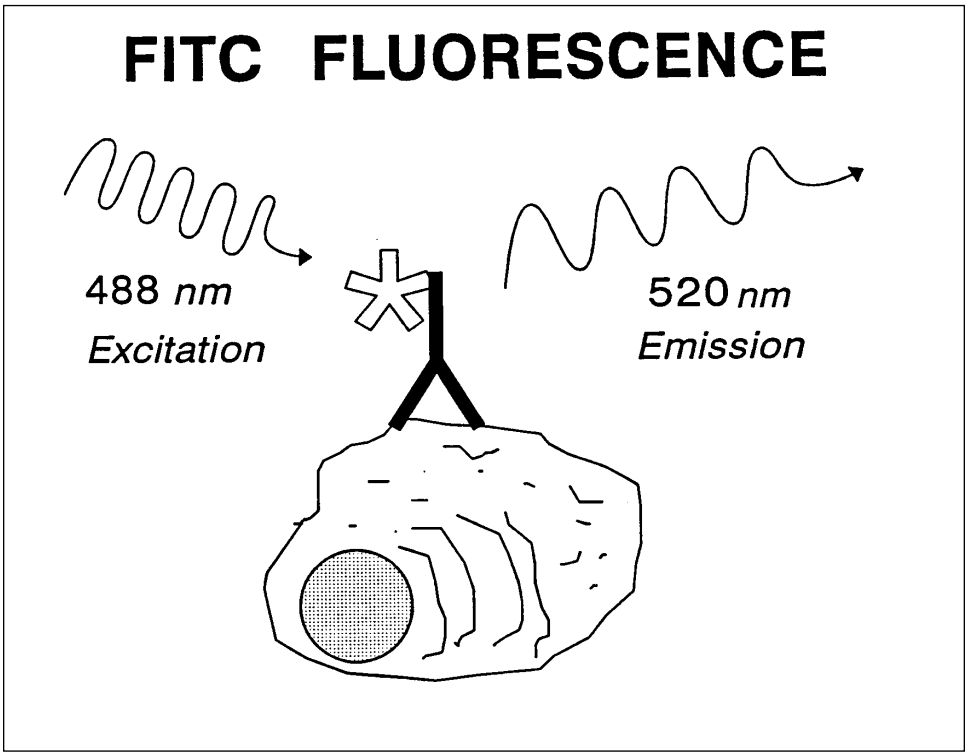


Figure 1. Diagram illustrating the excitation / emission of a FITC conjugated antibody. The excitation wavelength is 488nm (argon laser) and FITC emission occurs at 530mn.

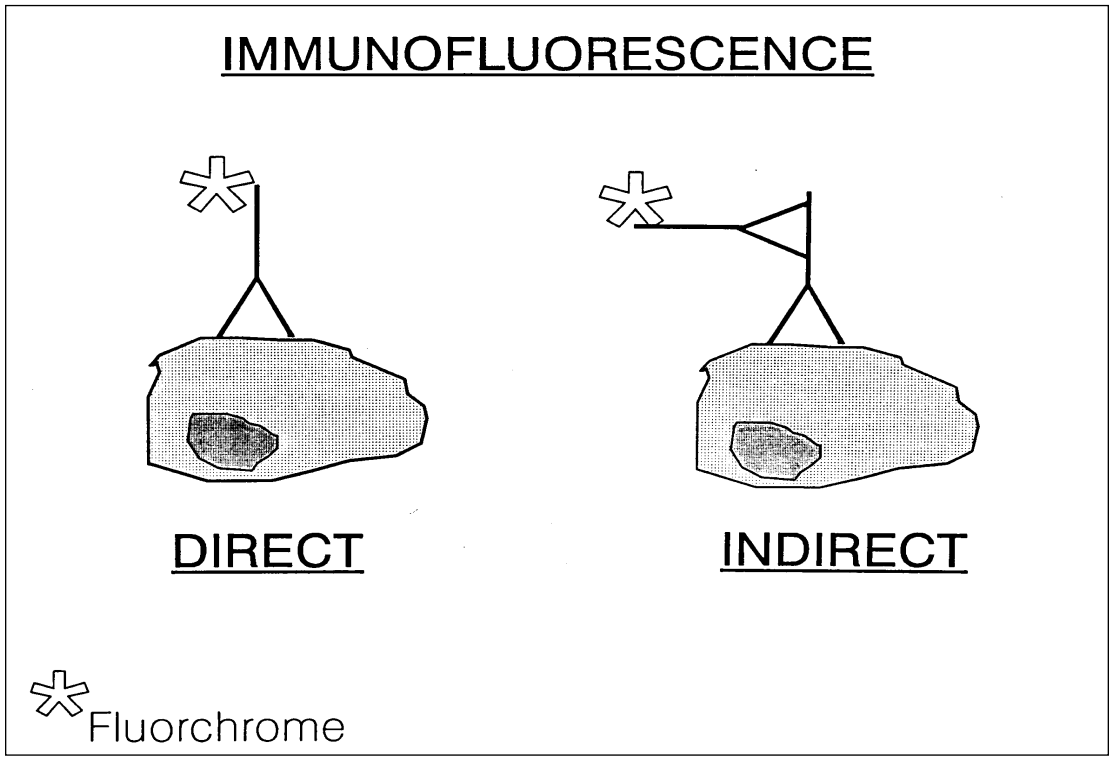


Figure 2. Illustration of Direct and Indirect Immunofluorescence.

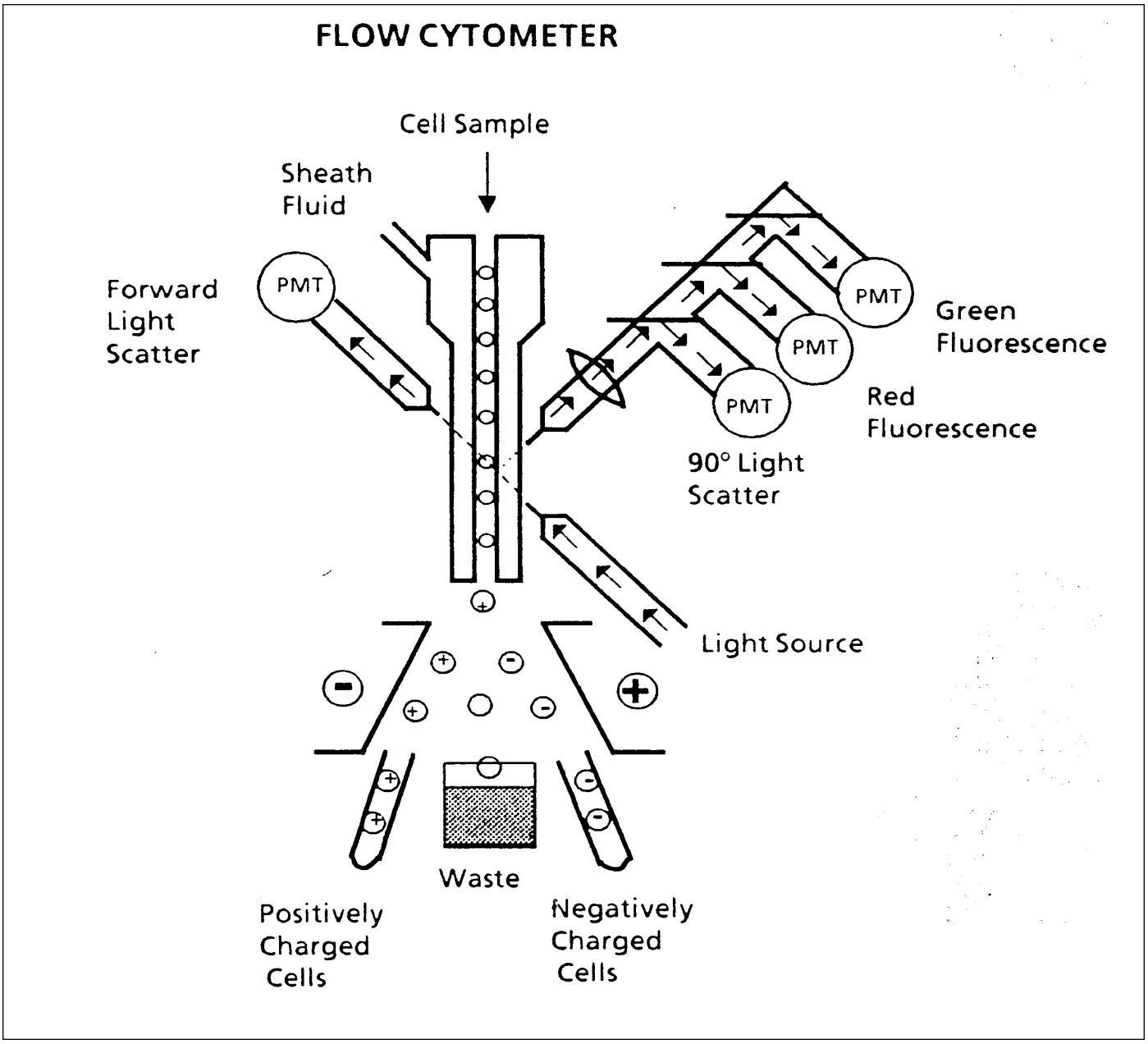


Figure 3. Schematic Diagram of a Flow Cytometer. PMT = Photomultiplier Tube. Reprinted by permission from: SEOPF Tissue Typing Reference Manual, JM MacQueen, ed.; Southeastern Organ Procurement Foundation, Richmond, VA,1987.

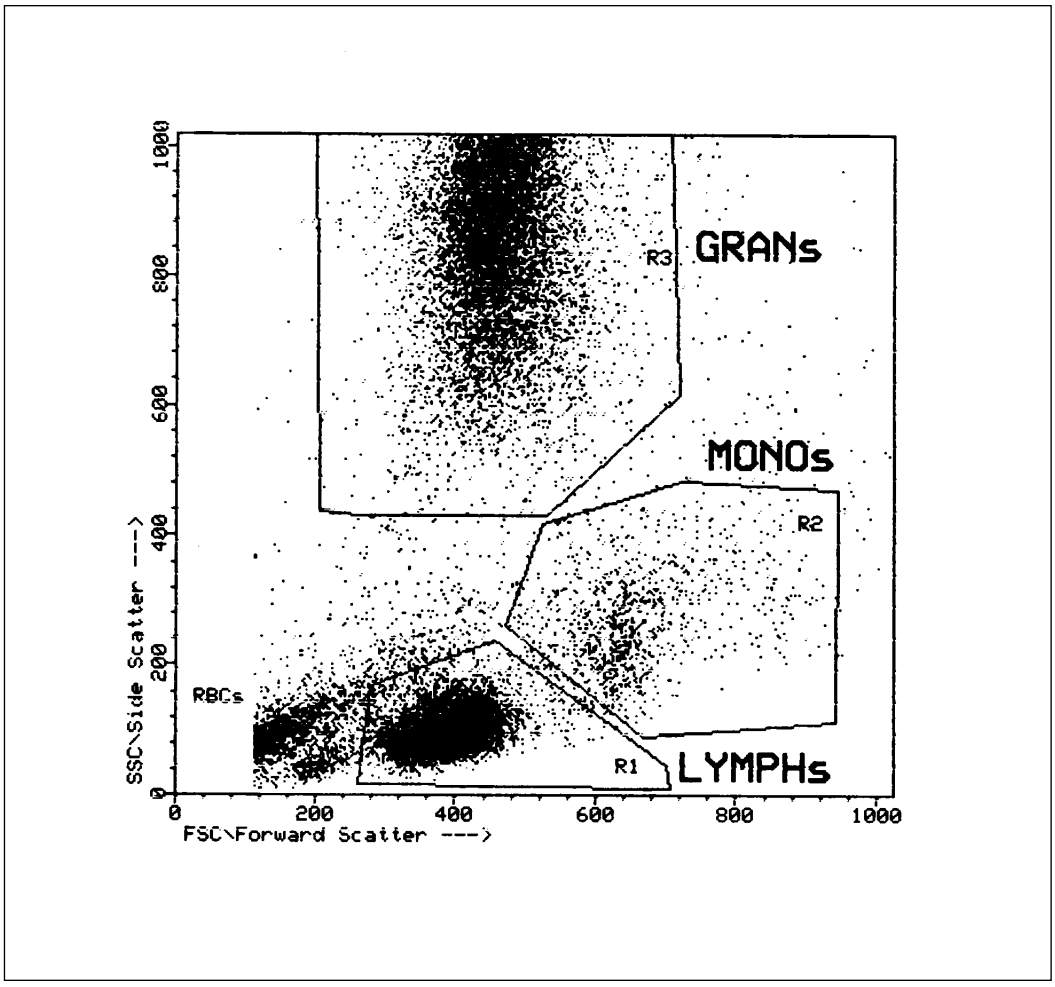


Figure 4. Illustration of forward angle light scatter (FSC; X-axis) versus orthogonal scatter (SSC; side scatter; Y-axis) of lysed whole blood. The diagram shows the indetification of 3 distinct cell populations based on light scatter only. Enclosed areas indicate the potential electronic "gates" that could be used for analysis. **Rl** = Lymphocytes; **R2** = Monocytes; and **R3** = Granulocytes. **RBCs** represent the area where unlysed red cells and debris will be found.

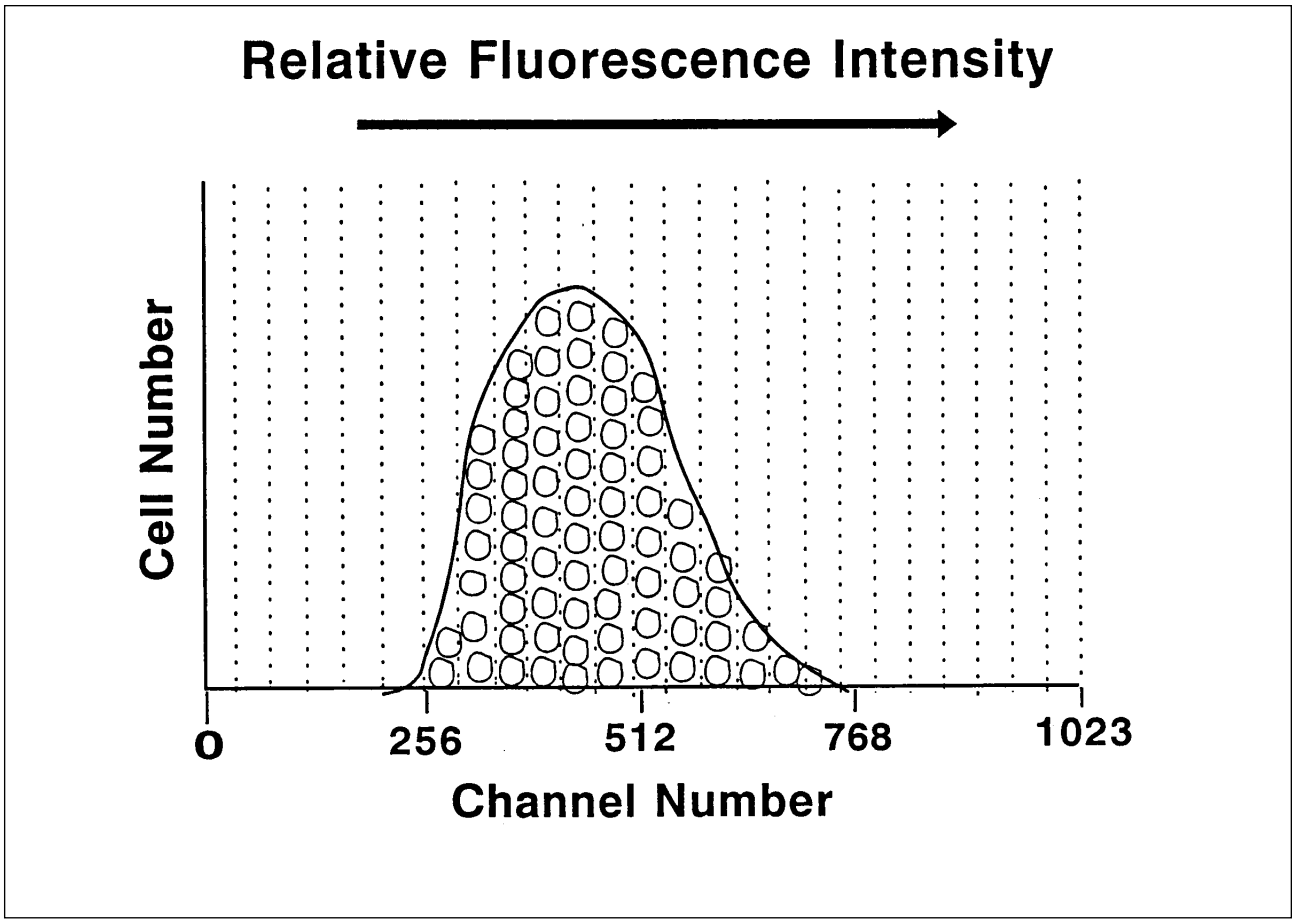


Figure 5. Illustration of a single parameter flow cytometric histogram depicting fluorescence intensity and channel values. X-axis depicts the individual channels where each channel reflects a different fluorescence intensity. Y-axis depicts the cell number. Diagram illustrates how data are collected to form a histogram as indicated by the shape of the curve.

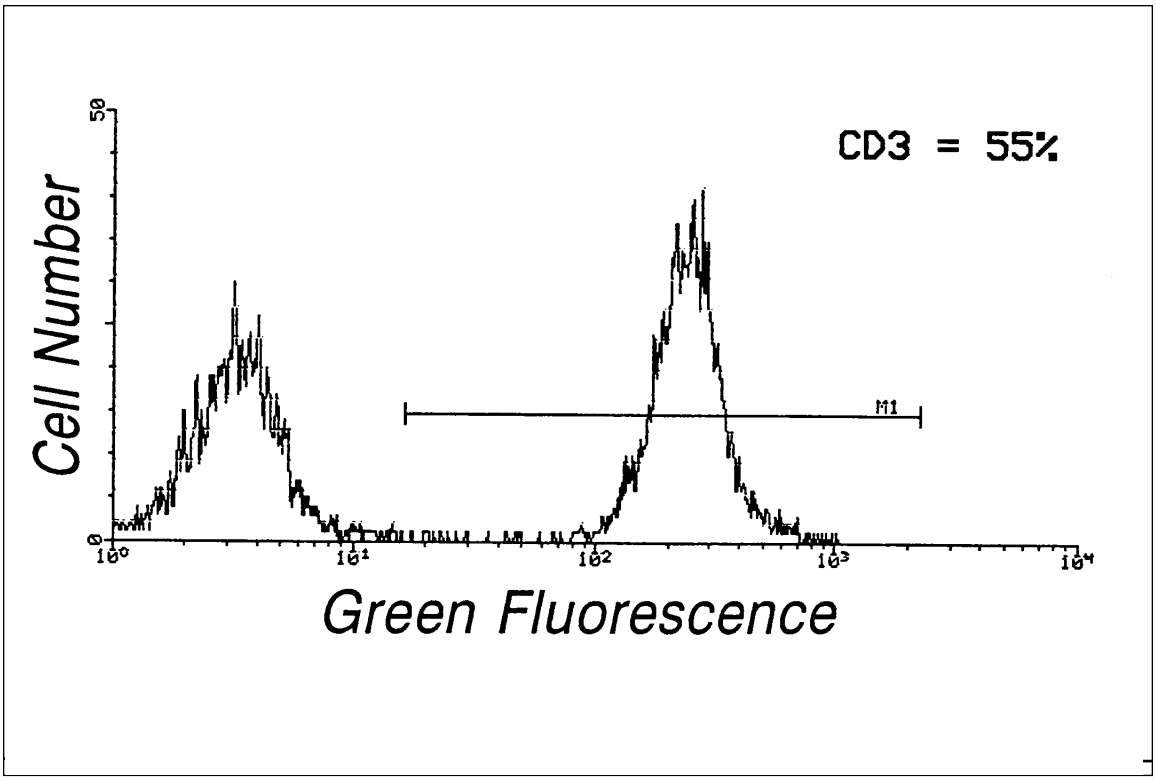


Figure 6. Single parameter frequency histogram of lymphocytes stained with FITC anti-CD3. The population to the left of the histogram represents the background fluorescence of CD3 negative cells. The population to the right on the histogram, indicated by the marker Ml, represents the fluorescence of the CD3 positive cells.

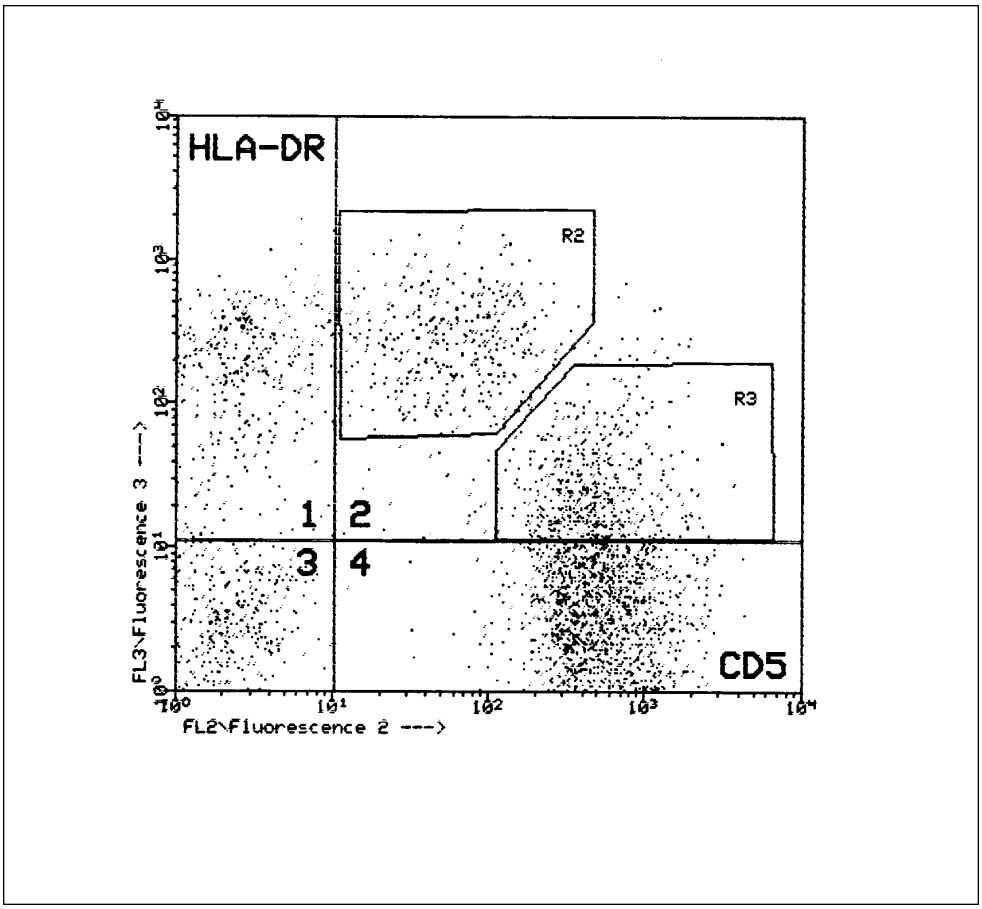


Figure 7. Two color immunofluorescence analysis of lymphocytes stained with PerCP' anti-HLA-DR (Y-axis) and PE CD5 (X-axis). The four quadrant settings were established from appropriate isotype controls. The regions, R2 and R3 represent subsets of B cells and T cells, respectively (see text).

Cell-based, Flow Cytometric Detection of Panel Reactive Antibody (FC-PRA): Setup, Acquisition of Data & Analysis

Lisa Wilmoth-Hosey, Pam Chapman, Joan Holcomb and Robert A. Bray

Purpose

The flow cytometric crossmatch (FCXM) is the most sensitive method for detecting anti-HLA antibodies in the sera of potential allograft recipients. The fact that the flow crossmatch is more sensitive than the AHG-CDC creates a situation wherein a CDC antibody screen may be negative but the FCXM final crossmatch is positive thereby precluding transplantation in certain instances. In order to better identify and define alloantibodies, the flow cytometric PRA (FC-PRA) using cell pools was developed to address routine antibody screening for selected patients. Such patients would include new transplant candidates who have a history significant for sensitization (i.e., multiple transfusions or pregnancies) and currently active patients in whom the antibody titer, by AHG-CDC, has significantly declined. The goal of performing a FC-PRA is to better determine the "sensitized" nature of a given patient. The bead based PRA can give a %PRA but specificity may not always be clearly identified. Hence, alternative methods for determining antibody specificity, at the same level of sensitivity as FCXM, are needed.

The FC-PRA is performed by using pools of well-characterized panel cells. The configuration discussed here utilizes 7 pools with 4 cells per pool (Figure 1). Each cell pool is constructed based upon CREG specificities, with an emphasis toward those prevalent in one's individual practice. The goal is to utilize patients whose private HLA antigens are contained within single CREGs (Table 1). Thus, a single pool, although comprised of many different private HLA antigens, tests for only 2 (1 A-locus and 1 B-locus) CREG. Thus, the nature of the pools are such that broadly reactive antibodies (CREGS) can be identified rather than multiple unique specificities. Patient's sera are tested undiluted against these pools and their reactivity patterns are recorded. From the reaction patterns, a percent PRA can be calculated and in many instances specificities can be assigned. The goal of the FC-PRA is to determine the presence or absence of alloantibodies in selected patients and to ascribe specificities, albeit broadly reactive (such as a CREG) to them.

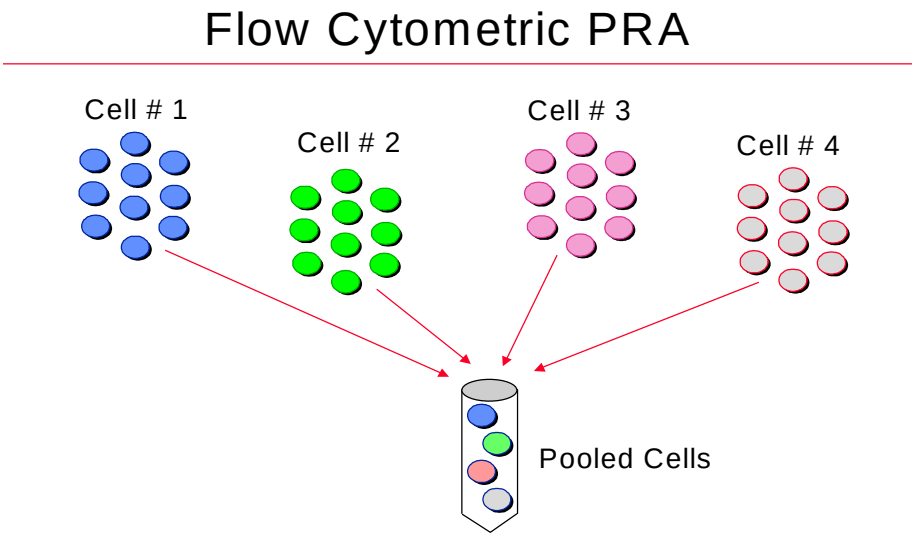


Figure 1. Illustration of how the FC-PRA pools are constructed.

Specimens

Serum. Minimum quantity needed is 250 µl.

Reagents, Supplies, Equipment

Negative control X2 (normal human and pooled human sera)
Positive control (pooled positive serum)
FITC-conjugated goat, anti-human IgG $F(ab)'_2$, Fc specific
(Jackson Labs, cat. #109-016-098)
Phycoerythrin Conjugated Anti-human CD3 antibody (Becton-Dickinson, Inc.)
Falcon tubes
RPMI + 20% FCS
Wash buffer (PBS, 2% FCS and 0.1% sodium azide, NaN_3)
2% paraformaldehyde in PBS, pH = 7.2 ±2
Eppendorf pipette & 8 channel attachment with appropriate tips
Brinkman transferpette™ -12 with appropriate tips
Pipetman with appropriate tips
Beckman airfuge with micro-ultracentrifuge tubes and protective caps
6 x 50 mm glass tubes (Baxter diSPo culture tubes, Cat. #T1290-1)
Beckman GP centrifuge; 96 well Costar Cell Culture Tray (Corning)
Flow Cytometer (FACScan, FACSort, or FACScaliber: Becton-Dickinson, Inc.)

Note: For those laboratories using a Coulter Flow Cytometer, this procedure may be used as a point of departure for doing cell based Flow PRA determinations. However, it must be validated for accuracy and reproducibility before actual patient testing and reporting.

Procedure

Flow PRA Setup

1. Serum Preparation:
   a. Pull patients sera, PPS and NHS, the current lots of Pel Freez lot #0706 and C-Six lot #960325 and allow to thaw at room temperature.
   b. Airfuge all patients sera and controls at 28 PSI for 10 minutes.
      – Total volume of 250 µl per sample is needed. (2 micro-ultracentrifuge tubes with 125 µl of serum in each).
2. Thaw frozen pool cells:
   a. Label the 15 ml conical tubes with corresponding colored tape (1 tube for each pool). The current number of pools is 7.
   b. Add 1 ml of RPMI-20% FCS to each tube.
   c. For each pool, pull the required volume of cells and place on dry ice in a Styrofoam container.
      Conc: 4×10^6/ml 2 vials for each pool
      8×10^6/ml 1 vial for each pool
   d. Thaw one pool at a time. Follow procedure for thawing of cells.
   e. Add the thawed cells to the correctly labeled 15 ml conical containing the RPMI with 20% FCS.
   f. To the tube of thawed cells slowly, drop by drop, add RPMI with 20% FCS until the tube is filled.
   g. Repeat steps **d-f** until all pools have been thawed.
3. Wash the cell pools:
   a. Place the 15 ml conical tubes containing the thawed cells in the centrifuge and spin for 1 minute at 2400 RPMs.
   b. Decant the supernatant and resuspend the cells in 10 ml of RPMI with 20% FCS.
   c. Repeat step 3a. for a second wash; decant the supernatant.
4. Cleanup of the flow PRA pool cells:
   Cleanup is usually not needed, but if viability is <70%, you may use a DNASE procedure for this purpose.
   Note: Do Not use Percoll or Lymphokwik to clean up cell prep
5. Label 6 ml falcon tubes, one for each pool, with the appropriate colored tape. To each of these tubes, add 2 ml of the flow wash buffer.
6. Transfer each cell prep. from the fisher tubes to the appropriately labeled 6 ml falcon tube.
7. Check the viability of all pools and perform a cell count.
8. Adjust the cell count to 2.5×10^6/ml with a minimum volume of 1.4 ml.
9. Take a 96 well tissue culture tray and label it with the setup date and the batch number.
10. Using an Eppendorf pipette, add 100 µl of each of the pool cells to the 96 well tray using the following format:

	1	2	3	4	5	6	7	8	9	10	11	12
A	<———					POOL 1						———>
B	<———					POOL 2						———>
C	<———					POOL 3						———>
D	<———					POOL 4						———>
E	<———					POOL 5						———>
F	<———					POOL 6						———>
G	<———					POOL 7						———>
H												

11. Replace the tray cover and spin the plate in the Beckman centrifuge (with the brake on) to pellet the pool cells. To spin, bring the centrifuge up to 900 xg (for 3 min.), then turn off.
12. Remove the tray from the centrifuge and flick the plate to remove the supernatant.
    To flick :
    Remove the tray cover and quickly turn the tray upside down so the supernatant is forcibly removed from the tray. Next, while still holding the tray upside down, place it on a layer of paper towels to pull the last remaining liquid from the wells.
13. Replace the cover and gently run the tray across a vortex in order to loosen the cell pellet.
14. To the tray, add patient serum and any controls using the following plating format: (read DOWN the columns)

	1	2	3	4	5	6	7	8	9	10	11	12
A	N	P	S	S	S	S	S	S	S	S	S	N
B	E	P	E	E	E	E	E	E	E	E	E	E
C	G	S	R	R	R	R	R	R	R	R	R	G
D	#	"	U	U	U	U	U	U	U	U	U	#
E	1	"	M	M	M	M	M	M	M	M	M	2
F	"	"	1	2	3	4	5	6	7	8	9	"
G	"	_	_	_	_	_	_	_	_	_	_	"
H												

15. Replace the tray cover and mix the pool cells and serum by gently running the tray over a vortex.
16. Incubate the tray for 30 minutes at 4°C.
17. Wash the tray:
    a. Remove the tray cover and with an eppendorf repeat pipette, add 75 µl of flow wash buffer to each well then vortex. Add an additional 75 µl to each well to complete the wash (do not vortex at this point since splash over may occur).
    b. Replace the tray cover and spin in the Beckman centrifuge (brake on) to pellet the cells. To spin, allow the centrifuge to reach 900 xg for 3 minutes, then turn off.
    c. Remove the tray cover and flick the plate to remove the supernatant. Turn plate upside down on a layer of paper towels in order to drain the remaining liquid from the wells.
    d. Replace the tray cover and gently vortex the plate to loosen the cell pellet.
18. Repeat steps **17 a-d** two more times for a total of 3 washes.
19. After the last wash, make sure the cell pellet is dry and the tray has been vortexed to loosen the cell pellet.
20. Using the Brinkman pipette, add 20 µl of properly diluted IgG-FITC to every well containing pool cells.
21. Replace the tray cover and vortex the tray to mix the cells and FITC. Incubate for 10 minutes at 4°C.
22. Using the Brinkman pipette, add 20 µl of properly diluted CD3 PE to every well containing pool cells.
23. Replace the cover and vortex the cell/reagent mixture. Incubate for 20 minutes at 4°C.
24. Wash the 96 well plate x3 as described in steps 17a-d.

25. After the final wash, make sure the cell pellet is dry and the plate has been vortexed to loosen the cell pellet.
26. Using the eppendorf repeater pipet, add 75 µl of flow wash buffer to all 96 wells. Replace the cover on the tray and gently vortex.
27. Using the eppendorf repeater pipette, add 75 µl of paraformaldehyde to each well containing a cell prep. Replace the cover and gently vortex.
    Note: The tray may be stored in the dark at 4°C for up to 3 days.
28. When ready to run the setup on the flow cytometer transfer the sample preps to 6 x 50mm glass tubes using a multi-channel pipette.
    Note: For users of FACS… instruments, the 6 x 50 mm tube fits into the 12 x 75 mm tube for running.

References

1. Bray RA, Sinclair DA, Wilmoth-Hosey L, Lyons, C Chapman P and Holcomb J. 1998. Significance of the flow cytometric PRA (FC-PRA) in the evaluation of patients awaiting renal transplantation. Hu. Immunol. 59(suppl. 1):121.
2. Bray, RA. 1998. Flow Cytometry in the Evaluation of Patients Awaiting Organ Transplantation. Cytometry (supplment 9):36.
3. Bray RA, Foulks C, Wilmoth L, Chapman P and Holcomb J. 1997. Comparison between antiglobulin-enhanced cytotoxicity, flow cytometry and GTI quick screen for the detection of HLA alloantibody. Hu Immunol. 55 (suppl 1):74.
4. Bray RA, Chapman PT, Sinclair DA, Tate CA, Wilmoth LA, Holcomb JE and Rodey GE. 1996. The Flow Cytometric PRA: Evaluation of Antibody Reactivity and Specificity using Cell Pools based on CREGs. Hu. Immunol. 49(suppl 1): 106.

Table 1: Example of two of the seven cell pools used in the FC-PRA. Note that, for the most part, the private HLA specificities are contained within restricted CREGs.

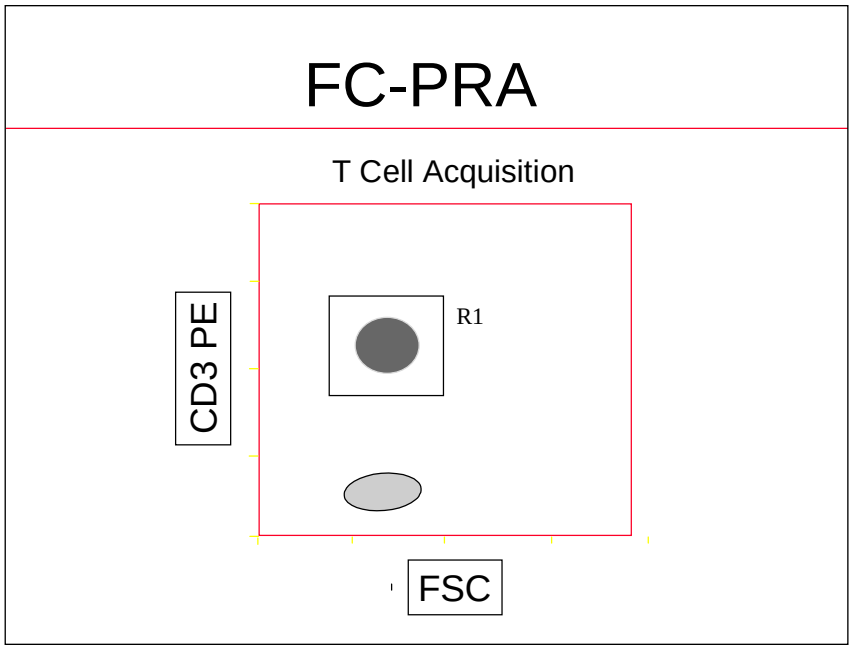
	Flow Cytometric PRA	
Pool #1	A2, A24; B13, B44	2C, 12C
	A2, A28, B44, B60	2C, 12C
	A68, A23, B44, B49	2C, 12C
	A2, A2, B45, B50	2C, 12C
Pool #2	A68, A69; B7, B7	2C, 7C
	A24, A24, B7, B56	2C, 7C
	A2, A28, B27, B13	2C, 7C
	A2, A28, B27, B60	2C, 7C

Acquisition of FC-PRA

For instrument setup, utilize the standard FACSCompTM or comparable validated instrument setup routine for your laboratory. The only difference between the FC-PRA acquisition and the FCXM acquisition is that it is only necessary to acquire T-cell events rather than total lymphocyte events. This can be done by gating only on the T-cell population (T-cell gate).

T-cell gate: This is accomplished by first setting a 2 parameter dot plot to display forward scatter (FSC) on the X-axis and fluorescence 2 (FL2; phycoerythrin) on the Y-axis (see figure 1.). This plot effectively selects for all T cells by virtue of their positivity for CD3. Next, set an acquisition gate (R1) around only those cells that are positive for FL2 (CD3). In addition, set one display box to reflect a histogram of FL1 from Region 1. After an acquisition gate has been determined, set the events to acquire to 10,000 and begin acquisition (see Figure 2 below).

FIGURE 2



Data Analysis

Analysis of FC-PRA is performed manually. Within each pool there are a total of four individual cells. Obviously, if all cells are positive or negative then the entire peak with either remains in the same position as the Negative control or shift to the right. However, if <4 cells are positive, then there will be some type of distinct "architecture" to assess. A sample is shown in Figure 3 below. To perform the analysis of all cell pools it is helpful to set up a single page printout as shown below (Figure 4). This figure shows 6/7 pools from a sample analysis. The HLA types of the individual cells contained within each pool is listed inside each histogram. If, from the pooled cells specificity cannot be clearly delineated, the individual cells from each pool may be run. See Figure 5 for an example.

FIGURE 3

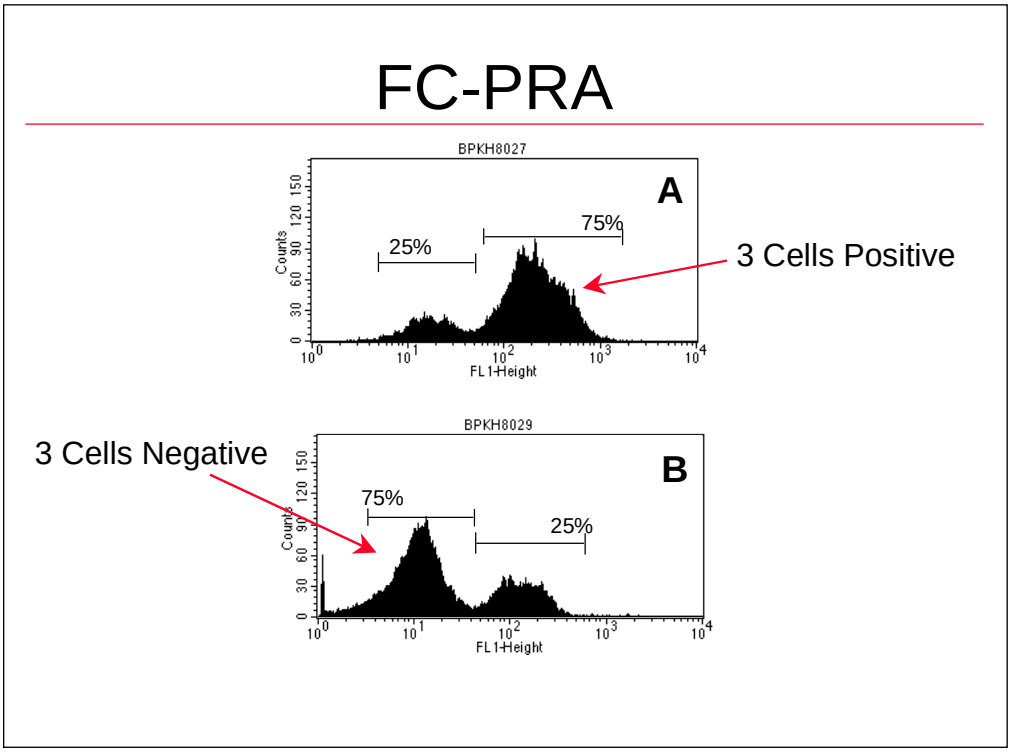


Figure 3. Illustrates examples of FC-PRA staining architecture. Histogram A depicts an example where 3 cell are positive and one cell negative, while example B illustrates where 3-cells are negative and one cell positive. In each example the indicated regions show the percentage of "Positive and Negative" events. Hence, 25% of the events would equal one cell.

FIGURE 4

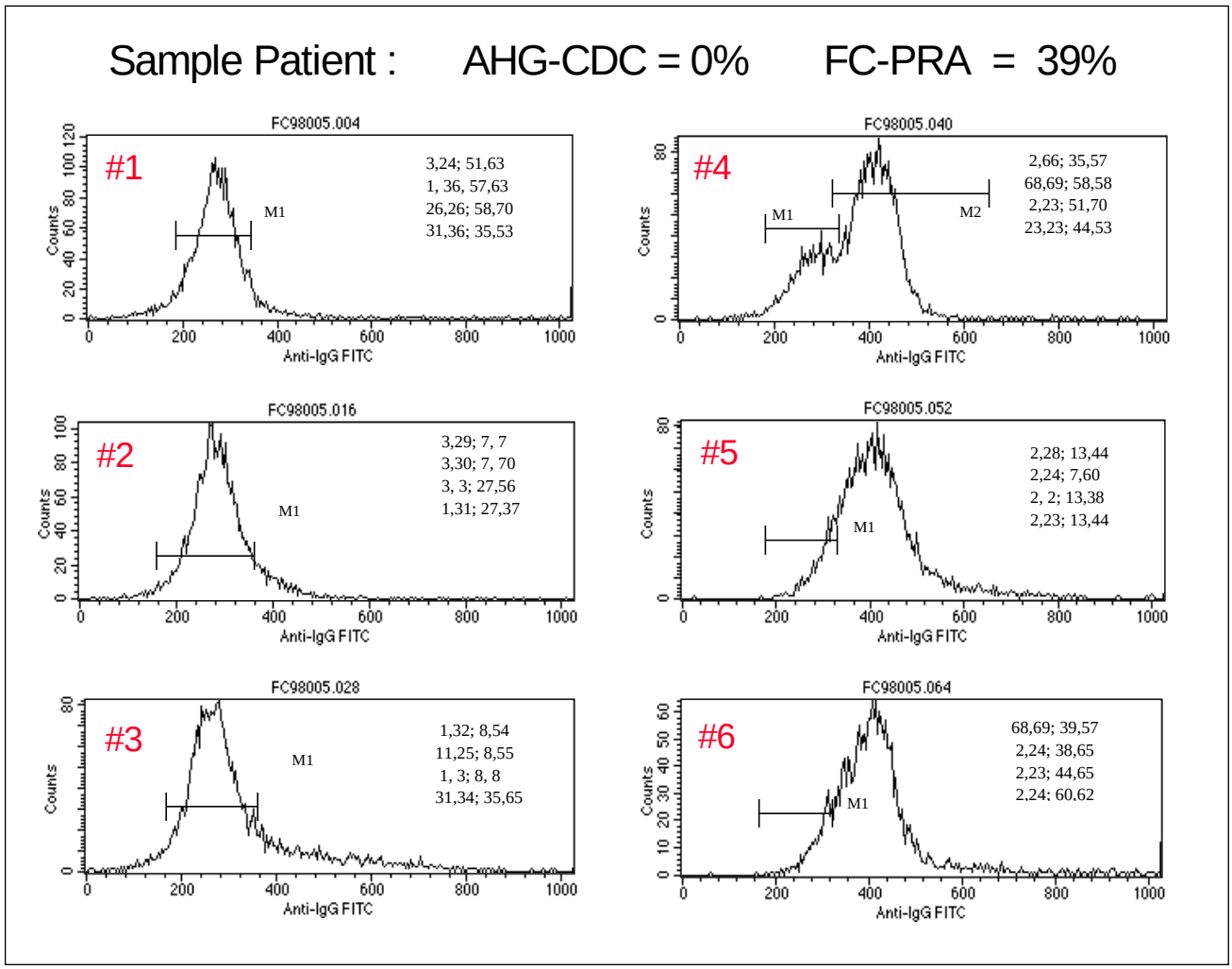


Figure 4. Sample analysis of a FC-PRA. The sample patient showed positivity with pools #4, 5 and 6. As the phenotypes indicate, each of these pools has an A2 CREG in common. The only outlier is seen in Pool #4 where there are 3 cells positive and one cell negative. The negative cell was shown to be the homozygous A23 cells. Thus, this antibody specificity would be A2, A28.

FIGURE 5

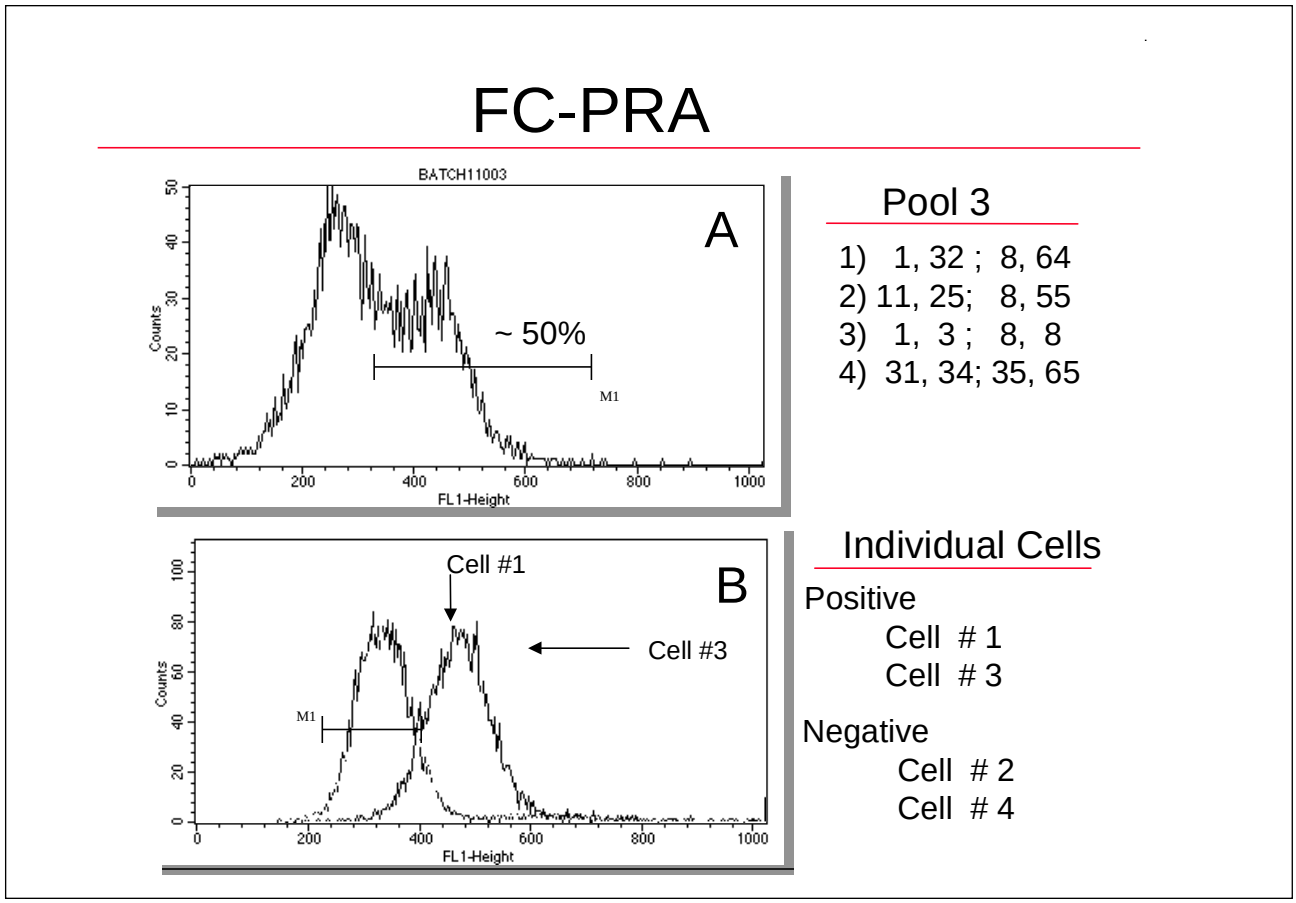


Figure 5. An example of a cell pool (A) and the individual cells run from that pool. As shown in A, two cells are positive (50%) and two cells are negative (50%). In some instances it may not be possible to discern which cells are positive and negative. For those instances, utilizing single cells may help determine specificity. As shown above, the two positive cells (#1 and #3) both carry A1. Interestingly, cell #3 has A1 and A3 and shows a channel displacement that is greater than Cell #1. This may indicate that the antibody reactivity is directed against a public epitope on A1 and A3 but not A11.

Antibody Detection by Flow Cytometry Using Antigen Coated Beads

Lisa Wilmoth-Hosey and Robert A. Bray

Purpose

Antibody detection and specificity identification have always played a major role in the function of an HLA laboratory. Any individual who has experienced sensitizing events such as transfusion, pregnancy or previous transplant is at risk for developing anti-HLA antibodies. Because of this it is important for the HLA laboratory to detect and identify these antibodies prior to the patient receiving an organ for transplant or retransplant.

In the past several years the sensitivity level for crossmatching has been significantly enhanced due to the use of the flow cytometer. However, in the area of antibody screening the sensitivity of detecting antibodies has remained confined within the limitations of the complement dependent cytotoxicity (CDC) assay. Increasingly, labs have been faced with the scenario of a patient having a PRA history of 0% by CDC and yet when a final crossmatch is set up with a potential donor the crossmatch is negative by CDC and positive by Flow. This example illustrates the importance of being able to screen for HLA antibodies using the same level of sensitivity as the crossmatch method.

In response to this need labs have been seeking a means of screening for antibodies using the flow cytometer. One avenue that has been pursued is the development of a flow PRA panel using known donor cells. In effect, this was an extension of the CDC assay concept only the method of detection was flow cytometry. While this has been effective, it is fairly time consuming to maintain the panel, and the setup and analysis are very labor intensive. Another method that has recently been made available to the transplantation community is the use of antigen coated latex beads to detect the presence of specific HLA antibodies. While the science of coating beads with antigen is one which has been time proven as an immunological technique, the application to HLA is new.

The HLA bead assay utilizes micro particles (2-4 μm in diameter) that have been coated with purified HLA antigen. Individual beads are coated with antigen from a single cell line then the beads are mixed together to form a pool of 30. The pool consists of the most common HLA antigens as well as some of the more infrequently seen types. While trying to cover the broad spectrum of antigens, the pool should also be representative of the frequency in which the antigens are seen in the population. Beads may be coated with either Class I or Class II antigen allowing for the simultaneous detection of either of these antibodies. Antibody screening using the flow bead methodology provides not only a positive or negative interpretation but also allows for the determination of a percent PRA. However, for assigning antibody specificity further testing is required.

Screening by flow cytometric methods has some obvious advantages over the CDC assay and using the beads to screen for antibodies has added advantages over cell panels. The flow cytometer has been shown to be more sensitive than the more conventional CDC assay and is also able to detect the presence of non-complement fixing antibodies which may be missed using a complement dependent test. In addition to the advantages ascribed to the methodology itself, flow beads are coated with 'purified' HLA antigen. Thus, excluding reactions, which could be attributed to non-HLA antibodies that are a problem when using cell panels. The flow bead assay also allows for the simultaneous detection of Class I and Class II antibodies and the patient sample required is minimal.

The flow bead screen is performed by incubating the HLA antigen coated beads with the test serum. Any antibody present in the test sample will bind to the beads during a brief incubation period and any excess serum is removed by a series of multiple washes. The addition of a Fluoresceinated (FITC) goat, anti-human immunoglobulin reagent allows for the detection of antibody attachment. Either IgG or IgM antibodies may be detected depending on the type of secondary antibody used. After the secondary antibody has been removed, samples are analyzed on the flow cytometer and results expressed as either negative or percent positive based on the shift in fluorescence intensity as compared to the negative control.

Specimen

Patient serum (either fresh or frozen) – 25 μl is needed for the test assay.

Ultracentrifugation of the sample is suggested prior to testing to remove aggregates and large immune complexes which may interfere with the assay.

Reagents and Supplies

Reagents

One Lambda Class I and/or Class II Flow PRA beads (cat.#FL12-60)
One Lambda flow bead wash buffer (working solution)(included in kit cat.#FL12-60)
Add 10 ml stock flow bead wash buffer to 90 ml distilled water (use at RT)
FITC conjugated goat, anti-human IgG [F(ab)'$_2$ Fc specific] (included in kit cat.#FL12-60)
or
FITC conjugated goat anti-human IgM [F(ab)'$_2$ Fc specific]
Negative control sera
Positive control sera

Source: One Lambda, Inc. (818)702-0042

Supplies:

Tube setup-used for low volume testing
Gel loading pipette tips
6x50 mm glass tubes (Fisher cat.#1496210M)
Gilson Pipetteman or similar adjustable pipette (adjustable volume 1-20 µl)
1-250 µl pipette tips (Robbins cat.#1017-01-1)

Tray setup-used for high volume testing
96 well U-bottom tissue culture tray (Costar cat.#3799)
Eppendorf multichannel pipettor
Reagent reservoir (Costar cat.#4870)
Eppendorf pipette tips (cat.#22-35-137-1)

General-
Polyethylene Micro ultra centrifuge tubes (Robbins cat.#1013-00-0
Eppendorf repeating pipette
Eppendorf Combitips plus 5 ml (cat.#22-26-640-3)
Eppendorf Combitips plus 0.5 ml (cat.#22-49-604-2)

Instrumentation/Equipment

Fisher table top centrifuge
Vacuum aspiration station
FACScan flow cytometer and MAC computer station (Becton Dickinson (800)448-2347)
or other flow cytometer(Coulter Epics XL, B-D FACScalibur, etc.) with a data management system
Airfuge
Beckman Table top centrifuge
Timer
Vortex
Room Temperature incubator

Calibration

The FACScan should be calibrated daily using Becton-Dickinson calbrite beads (Becton-Dickinson cat# 340486). Other flow cytometers should be calibrated daily according to the manufacturer's specifications.

Procedure

Test Setup

1. Thaw and mix all sera to be tested.
   Label the appropriate number of micro ultracentrifuge tubes and aliquot 100 µl into each tube.
   Airfuge all sera 10 min. at 28PSI
2. Label the appropriate number of 6x50mm glass tubes needed to run the test.
   Number 1 = NHS...normal human sera (negative control#1)
   Number 2 = PHS...pooled human sera (negative control#2)
   Number 3 = PPS...pooled positive control
   Number 4... onwards = patient samples

or

Label a 96 well tissue culture tray with the batch number.
Well A1 = NHS...normal human sera (negative control#1)
Well A2 = PHS...pooled human sera (negative control#2)
Well A3 = PPS...pooled positive control
Well A4 thru H12 = patient samples

3. Mix beads very well by vortexing until the beads are completely resuspended.
4. Using an Eppendorf repeating pipette, add 5 µl Class I and/or Class II beads to each of the above labeled tubes/or wells.
   Note: a Gilson Pipetteman or similar multichannel pipette with gel loading tips may also be used to dispense beads. Also, when testing both class I and II together the beads may be pooled and 10 µl of the bead mix may be added to the tubes/wells).
5. Using the Gilson Pipetteman/pipette tips add 25 µl of control or patient serum to the beads.
6. Vortex each tube or tray (replace cover) and incubate at room temperature for 30 minutes in the dark.
7. Wash samples
   Washing glass tubes (x2):
   a. Add 400 µl One Lambda flow bead wash buffer (room temperature) and vortex
   b. Spin at 6000 RPM for 1.0 min in the Fisher table top centrifuge.
   c. Aspirate the supernatant to a dry button (taking care not to aspirate the beads).
   d. Repeat.
   Washing 96 well tray (x3):
   a. Add 75 µl One Lambda flow bead wash buffer to each well. Use a multichannel adapter for the Eppendorf repeating pipette.
   b. Vortex the tray to mix the beads well. Cover tray to avoid splash over between wells).
   c. Add another 75 µl of flow bead wash buffer to the wells.
   Note: *Do Not* vortex at this point because of the risk of splash over between wells.
   d. Replace tray cover and spin in the Beckman table top centrifuge at 900G for 3 minutes with the brake on.
   e. Remove the tray from the centrifuge and "flick" the plate to remove the supernatant.
      To flick:
      Remove the tray cover and quickly turn the tray upside down so that the supernatant is forcibly removed from the wells. While still holding the tray upside down, place it on a layer of paper towels to pull the last remaining liquid from the wells.
   f. Replace the cover and gently run the tray across the vortex in order to loosen the bead pellet.
8. Add 20 µl anti-human FITC (IgG or IgM) to the dry button.
9. Vortex samples and incubate in the dark at room temperature for 30 min.
10. Resuspend in One Lambda flow bead wash buffer and vortex.
    Tubes: add 200 µl
    Tray: add 75 µl vortex and add another 75 µl.
11. Transfer the volume from the wells to pre-numbered 50x6mm glass tubes using a multichannel pipettor.
    Note: samples may be run immediately or fixed with a 1% paraformaldehyde solution and stored at 4°C up to 24 hours. To fix add equal volumes of the flow bead wash buffer and 1% paraformaldehyde and vortex.
12. The samples are now ready for flow cytometric analysis. Specific instructions may vary according to the type of instrument used but the general concept will be the same.
    Note: Following is a general discussion on acquisition as well as specific directions for BD FACScan instrument running the Macintosh/CELLQuest program. This procedure may be used as a point of departure for other instruments, but must be validated prior to actual patient testing.

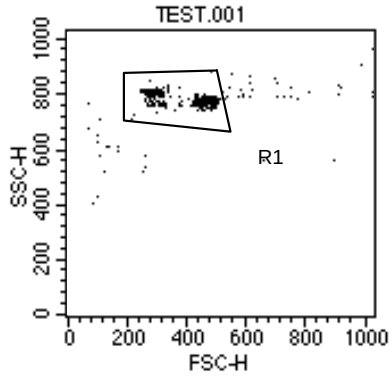
FACScan ACQUISITION and Analysis

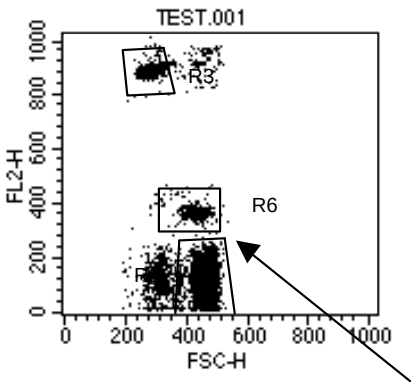
13. Start up the flow cytometer and perform required daily maintenance and calibration.
14. Open CELLQuest acquisition program
15. Open a template for running flow beads or set up screen for bead acquisition as appropriate.
    Figure 1 depicts a sample template showing a negative control serum.

NHS negative control

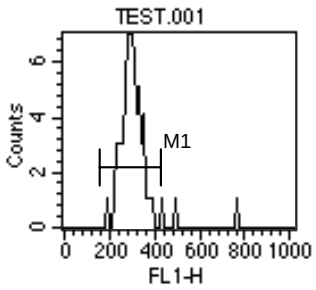
CLASS I: _____
CLASS II: _____

TECH _____
DIRECTOR _____



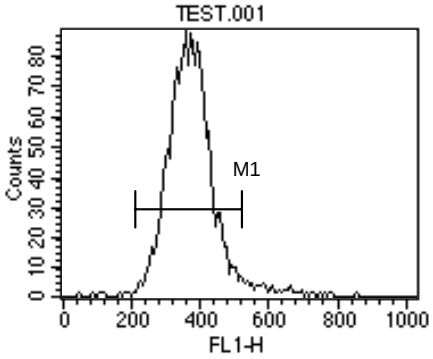


NSA CONTROL BEADS



R6= region gate for control beads

CLASS I



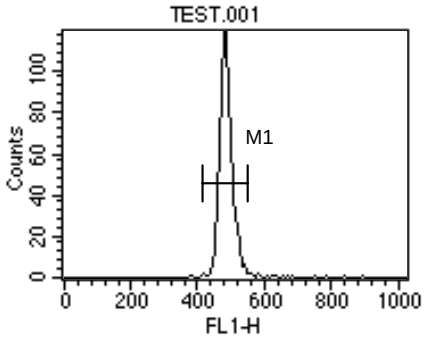
Histogram Statistics

File: TEST.001
Sample ID: CLASS I / II LOT#
Acquisition Date: 30-May-00
Gated Events: 9850
X Parameter: FL1-H (Log)

Log Data Units: Channel Values
Patient ID: CONTROL BDS LOT # 2
Gate: G4
Total Events: 15882

Marker	Left, Right	Events	% Gated	% Total	Median	Peak Ch
All	0, 1023	9850	100.00	62.02	364.00	353
M1	211, 521	9680	98.27	60.95	363.00	353

CLASS II



Histogram Statistics

File: TEST.001
Sample ID: CLASS I / II LOT#
Acquisition Date: 30-May-00
Gated Events: 3954
X Parameter: FL1-H (Log)

Log Data Units: Channel Values
Patient ID: CONTROL BDS LOT # 2
Gate: G5
Total Events: 15882

Marker	Left, Right	Events	% Gated	% Total	Median	Peak Ch
All	0, 1023	3954	100.00	24.90	476.00	470
M1	416, 552	3917	99.06	24.66	476.00	470

Figure 1. Acquisition and analysis template.

16. Instrument settings may need to be adjusted to bring the beads (which are small in size) on screen.
    Change the FSC detector voltage from E00 to E01.
    Change the SSC detector mode from LIN to LOG.
    Decrease the SSC detector voltage to 250 or until the beads appear on the FSC/SSC dot plot.
    Set the instrument to collect 15,000 R1 gated events.
    Set the log data units to channel values.
17. Setup folder and file names for data storage.

18. Place the flow in Setup and put the Negative control tube on to run. Fine adjustments should be made in the FSC/SSC and FSC/FL2 dot plot region gates for the Class I and II beads (Fig.1). Once adjusted on the negative control for a given setup then the region gates should not need to be moved. When adjustments are complete remove the instrument from setup and acquire the data for the negative control.
    R1 sets a general gate around the Class I (population on the right) and Class II beads (population on the left).
    R2 gates specifically around the Class I bead population.
    R3 gates specifically on the Class II bead population.
19. Once acquisition is complete set a negative marker around each of the Class I and Class II histogram peaks. Once the negative marker is set DO NOT move it during the running and analysis of additional samples (Fig.1).
20. Print the report which should contain the dot plots, histograms and relative statistics as well as patient identification (Fig.1).
21. For each subsequent tube run the sample as described above, however on analysis DO NOT change the M1 region that was drawn for the negative control. After acquisition is complete leave the M1 region where it is and draw a new region (M2) around the patients positive bead population. Refer to Result section for description of positive bead populations.
22. Print reports for each patient tested as well as controls.

Results

With the flow bead screening assay a percent PRA is determined for each patient. Any shift in the bead peak as compared to the negative control is indicative of antibody attachment and subsequent detection. Since the test consists of a pool of beads each with different HLA antigens attached some beads may remain negative while others shift to the right and are positive. The percent PRA is determined by the percentage of beads that have been gated as positive in the Class I and Class II histograms (M2 marker). This % gated is printed on the final report in the histogram statistics box. In theory, analysis and interpretation of flow screening results should be simple. An antibody attaches to a bead, FITC tags the alloantibody that results in a shift of the bead peak on the flow cytometer. As with most things the line between theory and clinical application is blurred. Some patients exhibit a distinct separation between the negative and positive bead populations. Others show only a slight separation and may actually be merged with the negative bead population. Plus, to confuse the issue even more some patients that are negative may actually fall to the right of the negative control marker. Experience is the key when interpreting the results of patient results that do not happen to fall into the area of distinct separation. Following are a few representative samples of flow bead screens that you may encounter.

The first example is a negative screen (Fig.2). This patient has only one peak, which should be similar in shape to the negative control and will usually fall within the negative control marker. On occasion this one peak may stray to the left or right of the negative control marker but still be interpreted as negative. This may possibly be due to protein concentration in the patient sample that is either lower or higher than the negative control used. The key is that the peak shifts as one entity without any variation in the architecture displayed by the negative control.

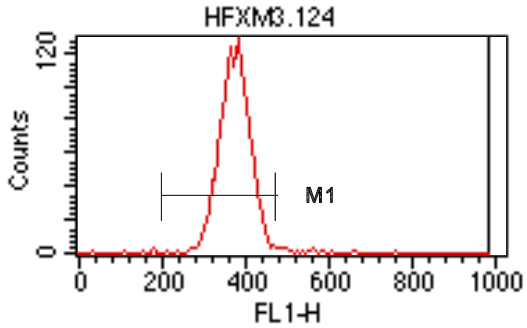


Figure 2. Sample of patient with no panel reactive antibodies.

There are also samples which show a change in peak architecture yet ancillary peaks are still within the region of the negative control marker and may actually be merged with the negative bead population (Fig.3). The approach to analyzing samples such as these is to look for any demarcation in the positive and negative populations, especially along the top ridge of the peak and set the positive bead marker accordingly. It is important to keep in mind that some patients falling within this group may also have a high background due to high protein concentration and accordingly the negative peak may shift outside of the negative control marker (Fig.4). The analysis strategy for this type of sample is the same as above (Fig.3), once the negative peak has been distinguished.

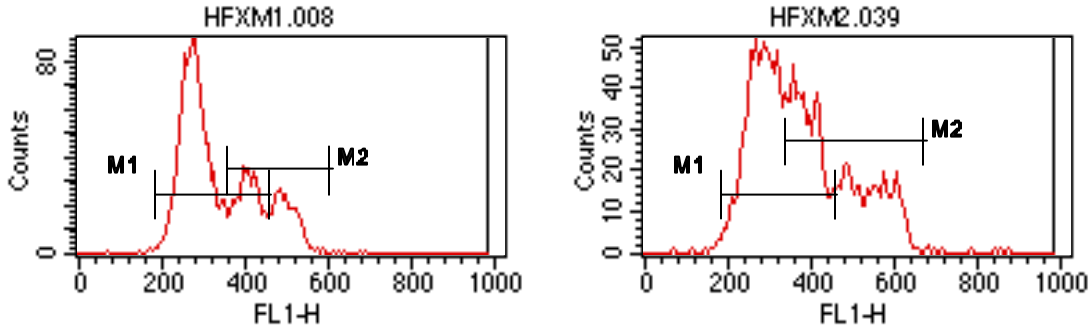


Figure 3. The histogram on the left is an example of a flow bead screen where the majority of the beads remained negative (the first peak) and some of the beads shifted to the right (peaks2 and 3). Even though the second peak is within the negative marker M1 it is considered positive since it has separated out from the first peak. The histogram on the right shows a patient sample which is positive (M2) and where the positive beads are merged with the negative bead peak.

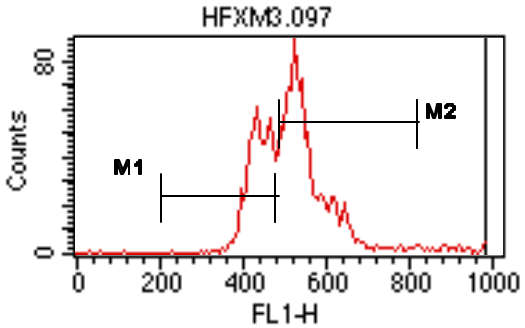


Figure 4. This histogram depicts a patient with a high fluorescent background. The negative peak is shifted to the right of the negative marker M1 and the patient also displays a positive peak M2.

Finally, there are the samples that show a distinct separation of the positive and negative peaks (Fig.5). These samples are the easiest ones to analyze and provide the most accurate %PRA due to the fact that there is no merging of the negative and positive bead populations.

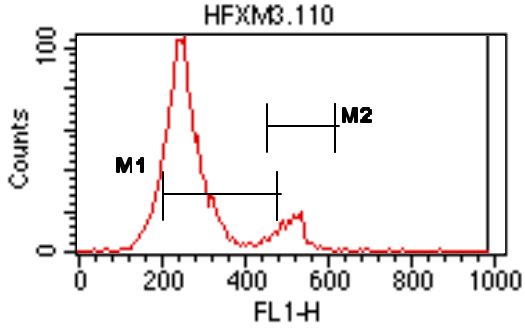


Figure 5. Histogram for a positive patient sample (M2). The positive population is completely shifted away form the negative marker and peak

Care should be taken when interpreting results on patient samples that are badly hemolyzed or contaminated with bacteria (Fig.6). Results may be invalid in these situations, especially if aggregates have occurred in the serum. Although it is unclear how severely hemolyzed samples effect the results, bacterial contamination have been demonstrated to cause a false positive interpretation. The test with a must be repeated with a sample that has been HI and airfuged for 30 minutes.

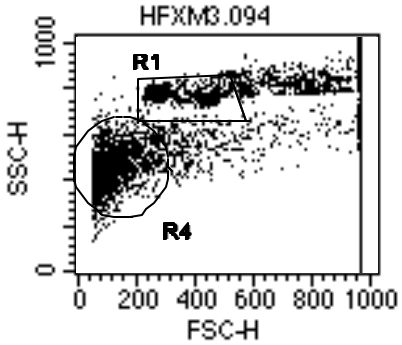
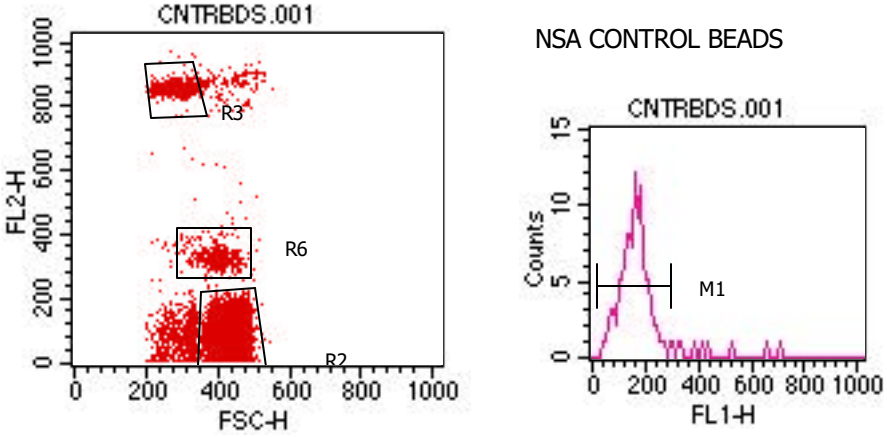


Figure 6. Example flow analysis of a specimen contaminated with bacteria (region R4).

Problem Samples

On occasion, some samples may exhibit a shift in the bead population to the right of the NHS control. This shift is as a single peak and may not be due to antibodies directed against HLA antigens on the bead surface. When this occurs, it is informative to retest the sera with the One Lambda Control beads. The control beads consist of the same latex bead as the Class I/II pool screening beads but are not coated with HLA antigen. Instead, they are coated with human serum albumin (HSA). The modifications to the Flow PRA bead protocol required for this procedure are as follows:

- Add the pool Class I/II beads to the glass tubes or trays as normal.
- Mix the control beads well by vortexing.
- Add 1 µl of the control beads to NHS, PPS and any patient samples to be tested..
- Follow the routine flow bead procedure for incubation times, washes and addition of FITC reagent.
- For FACScan Acquisition and analysis use the template seen in Figure 1. This template contains an additional R6 region within the FSC-FL2 dot plot that gates around the control bead population. In addition, a control bead histogram has been added to view the shift in FITC fluorescence attributed to the control bead itself rather than to any HLA specific antibody.
- For the control bead histogram, place a marker around the NHS sample and do not move the marker. No other markers need to be set in this box.
- For patient samples evaluate this box for any shift in the control bead peak to the right of the NHS control peak.



- Should there be a shift in the patient HSA control bead peak, this would indicate the shift in the FITC fluorescence and can be attributed to non-HLA factors. This should be taken into consideration when evaluating the Class I and Class II bead results, especially class I/II results that have been interpreted as positive and have shifted as one single peak. Note that a patient may have a shift in the control beads due to non-HLA factors and still have an additional shift of bead populations in the Class I and II histogram boxes that is a relevant HLA antigen-antibody reaction.

References

1. Bray RA, Cook DJ, Gebel HM. Flow cytometric detection of HLA alloantibodies using Class I coated microparticles. Human Immunol. 55:36, 1997.
2. Pei R, Wang C, Tarsitani S, et al: Simultaneous HLA Class I and Class II antibody screening with flow cytometry. Human Immunol. 59:313-322, 1998.
3. One Lambda, Inc., Flow PRA Screening Test package insert,. One Lambda, Inc., Canoga Park CA, 1998

Antibody Identification by Flow Cytometry Using HLA Class I or Class II Antigen Coated Specificity Beads

Lisa Wilmoth-Hosey and Robert A. Bray

Principle / Purpose

Antibody detection and specificity identification have always been a major function of an HLA laboratory. Any individual who has experienced sensitizing events such as transfusion, pregnancy or previous transplant is at risk for developing anti-HLA antibodies.

Because of this, it is important for the HLA lab to detect and identify these antibodies prior to the patient receiving an organ for transplant or retransplant.

In the past several years the level of sensitivity for crossmatching has been significantly enhanced due to the use of the flow cytometer. However, in the area of antibody screening the sensitivity of detecting antibodies has remained confined within the limitations of the complement dependent cytotoxicity (CDC) assay. Increasingly, labs have been faced with the scenario of a patient having a PRA history of 0% by CDC and yet, when a final crossmatch is set up with a potential donor, the crossmatch is CDC negative but positive by flow cytometry. This example illustrates the importance of being able to screen for HLA antibodies using the same level of sensitivity as the crossmatch method.

In response to this need, labs have been seeking a means of screening for antibodies using the flow cytometer. One avenue that has been pursued is the set up of a flow PRA panel using known donor cells. This in effect was an extension of the CDC assay concept only the method of testing was flow cytometry. While this has been effective it is fairly time consuming to maintain the panel, and the setup and analysis are very labor intensive. Additionally, and as a result of the increased sensitivity of flow cytometry, a positive result with cells may not always be due to HLA antibody. An alternative method that has recently been made available to the transplantation community is the use of antigen coated latex beads to detect the presence of HLA antibodies. While the science of coating beads with antigen is one which has been time proven the application to HLA is new.

Briefly, the specificity bead assay utilizes micro particles which have been coated with purified HLA antigen. Individual beads are coated with antigen from a single cell line then 8 groups of beads are mixed together to form a pool. A total of 4 pools are used for testing, providing a panel size of 32. The pools should consist of the most common HLA antigens as well as some of the more infrequently seen types. While trying to cover the broad spectrum of antigens the pools should also be representative of the frequency in which the antigens are seen in the population.

Beads may be coated with Class I or Class II antigen allowing for the determination of either of these antibodies. Antibody screening using flow specificity beads provides not only a positive or negative interpretation but allows for the determination of a percent PRA as well as assignment of antibody specificity (ClassI or ClassII depending on the tests setup).

Testing by flow cytometric methods has some advantages over the CDC assay and using the beads to test for antibodies has some added advantages over flow cell panels. The flow cytometer has been shown to be more sensitive than the more conventional CDC assay and is also able to detect the presence of non-complement fixing antibodies which may be missed using a complement dependent test. In addition to the advantages ascribed to the methodology itself the flow beads are coated with 'purified' HLA antigen. Thus, excluding reactions that could be attributed to non-HLA antibodies which are a problem when using cell panels. The flow specificity bead assay allows for the determination of either Class I or Class II antibodies independent of one another and the patient sample required for testing is minimal, 100 µl for specificity beads as compared to 300 µl for ELISA assays.

The flow specificity bead test is performed by incubating HLA antigen coated beads with the test serum. Antibody present in the test sample will bind to the beads during a brief incubation period then any excess serum is removed by a series of multiple washes. The addition of a Fluoresceinated (FITC) goat, anti-human immunoglobulin reagent allows for the detection of antibody attachment. Either IgG or IgM antibodies may be detected depending on the type of secondary antibody used. Once the secondary antibody has been removed, the samples are analyzed on the flow cytometer and the results for each bead are expressed as either negative or positive. This reaction assignment is based on the shift in fluorescence intensity as compared to the negative control populations. Antibody specificity is then determined based on pattern analysis of the positive reactions for a given sample.

Specimen

Patient serum (either fresh or frozen)....100 µl is needed for the test assay.

Ultracentrifugation of the sample is suggested prior to testing to remove aggregates and large immune complexes which may interfere with the assay.

Reagents and Supplies

Reagents

One Lambda FlowPRA Specific™ Class I beads (cat#FL1SP)
or
One Lambda FlowPRA Specific™ Class II beads (cat#FL2SP)
One Lambda flow bead wash buffer (working solution)(included in specificity bead kits)
Add 10 ml stock flow bead wash buffer to 90 ml distilled water (use at RT)
FITC conjugated goat, anti-human IgG [F(ab)'$_2$ Fc specific] (included in kit)
or
FITC conjugated goat anti-human IgM [F(ab)'$_2$ Fc specific]
Negative control sera
Positive control sera

Source: One Lambda, Inc. (818)702-0042

Supplies

Tube setup- used for low volume testing
Gel loading pipet tips
6x50 mm glass tubes
Gilson pipetman (adjustable volume 1-20 µl)
1-200 µl pipet tips

Tray setup- used for high volume testing
96 well tissue culture tray
Eppendorf multichannel pipeter
Reagent reservoir
Eppendorf multi channel pipet tips

General-
Polyethylene Micro ultra centrifuge tubes
Eppendorf repeater pipet
Eppendorf pipet tips
Eppenforf Combitips plus 5 ml
Eppendorf Combitips plus 0.5 ml

Instrumentation/Special Equipment

Fisher table top centrifuge
Vacuum aspiration station
FACScan flow cytometer and MAC computer station
Airfuge
Beckman Table top centrifuge
Timer
Vortex
Room Temperature incubator

Calibration

The FACScan should be calibrated daily using Becton-Dickinson calbrite beads.
(Becton-Dickinson cat# 340486)

Procedue

The flow PRA screening beads detect the presence of HLA antibody but in order to identify antibody specificity additional testing using flow specificity beads is required. The specificity beads are similar to the screening beads in that they are coated with HLA antigen and the test is performed by incubating the beads with patient sera, followed by the addition of fluoresceinated (FITC) anti-human immunoglobulin reagent. The added advantage of the specificity beads is that instead of consisting of one pool of thirty beads they are 4 different pools with each pool containing only 8 beads (for a panel size of 32). Within each pool the individual beads exhibit different fluorescent properties. This characteristic allows for the determination of which specific bead within the pool is positive or negative as compared to the negative control.

Since the HLA typing of each bead is known antibody specificity can be determined based on pattern analysis of the positive reactions.

1. Thaw and mix all sera to be tested.
   Label the appropriate number of micro ultracentrifuge tubes and aliquot 150 µl into each tube.
   Airfuge all sera 10 min. at 28PSI
2. Label the appropriate number of 6x50mm glass tubes needed to run the test.
   For each pool (total of 4) of specificity beads the following tubes will be needed
   Number 1 = NHS (Negative control #1)
   Number 2 = PHS (Negative control #2)
   Number 3 = PPS (Positive control)
   Numbers 4 onwards = patient samples

-OR-

   Label a 96 well tissue culture tray with the batch number.
   The bead pools will be plated in the rows while serum will be
   plated in columns. (See Figures 1 and 2).
3. Mix each group (1-4) of beads very well by vortexing until the beads are completely resuspended.
4. Using an Eppendorf repeater pipet, add 5 µl of flow specificity beads (Class I or Class II) to each of the above labeled tubes/or wells (see fig. 1 for tray format) (Note: a Gilson pipetman with gel loading tips may be used to dispense the beads into the glass tubes.)

Figure 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	<———					POOL 1						———>
B	<———					POOL 2						———>
C	<———					POOL 3						———>
D	<———					POOL 4						———>
E												
F												
G												
H												

5. Add 25 µl of control or patient serum to each pool of beads.
   If using tubes follow the format as listed for each pool of beads (1-4):
   Add serum using a gilson pipetman.
   Number 1 = NHS
   Number 2 = PHS
   Number 3 = PPS
   Numbers 4 onwards = patient samples

   If using 96 well tray see fig. 2 for format. Sera may be added using the eppendorf multichannel pipeter.
   Samples are added to rows A-D only.

Figure 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	N	P	S	S	S	S	S	S	S	S	S	P
B	H	P	E	E	E	E	E	E	E	E	E	H
C	S	S	R	R	R	R	R	R	R	R	R	S
D			U	U	U	U	U	U	U	U	U	
E			M	M	M	M	M	M	M	M	M	
F			1	2	3	4	5	6	7	8	9	
G												
H												

6. Vortex each tube or the tray (replace cover) and incubate at room temperature for 30 minutes in the dark.
7. Wash samples (x2 for tubes / x3 for 96 well tray)
   Washing glass tubes:
   a. Add 400 µl One Lambda flow bead wash buffer and vortex (use buffer at room temperature).
   b. Spin at 6000 RPM for 1.0 min in the Fisher table top centrifuge.
   c. Aspirate the supernatant to a dry button (be careful not to aspirate the beads).
   d. Repeat.
   Washing 96 well tray:
   a. Add 75 µl One Lambda flow bead wash buffer to each well. (Use a multi channel adapter for the Eppendorf repeater pipet.)
   b. Replace cover and vortex the tray to mix the beads well.
   c. Add another 75 µl of flow bead wash buffer to the wells. Do NOT vortex at this point because of the risk of splash over between wells.
   d. Replace tray cover and spin for 3 min. at 900g with the brake on low.
   e. Remove the tray from the centrifuge and flick the plate to remove the supernatant.
      To flick:
      Remove the tray cover and quickly turn the tray upside down so that the supernatant is forcibly removed from the well. While still holding the tray upside down, place it on a layer of paper towels to pull the last remaining liquid from the wells.
   f. Replace the cover and gently run the tray across the vortex in order to loosen the bead pellet.
   g. Repeat wash two more times.
8. Add 20 µl anti-human IgG FITC to the dry button.
9. Vortex samples and incubate in the dark at room temperature for 30 minutes. (Can use room temperature incubator.)
10. Wash samples (x2 tubes...x3 trays) as in step number 7.
11. Resuspend in One Lambda flow bead wash buffer and vortex.
    Tubes: add 200 µl
    Tray: add 75 µl vortex and add another 75 µl.
    Transfer the volume from the wells to pre numbered 50x6mm glass tubes using the eppindorf multi channel pipetter.
    NOTE: samples may be run immediately or fixed with a 1% paraformaldehyde solution and stored at 4°C up to 24 hours.
    To fix add equal volume of the paraformaldehyde to the flow bead wash buffer already in the tubes and vortex.
12. The samples are now ready for analysis on the flow. Specific instructions may vary according to the type of instrument used but the general concept will be the same. Following is a general discussion on the acquisition as well as specific directions for a BD FACScan instrument running the Macintosh/Cellquest program.

FACScan Acquisition and Analysis

13. Start up the flow cytometer and perform daily maintenance as required.
14. Open CELLQuest acquisition program.
15. Open a template for running flow beads or set up screen as required for specificity bead acquisition.
    See Figure 3 for a sample template showing a negative control serum.

The template used for data acquisition should be an acquisition template only. A comprehensive printout of each patient result will be generated at the time of analysis.

Data points are acquired by gating around the bead population in the FSC/SSC dot plot, region R1. R1 then provides the data which is displayed in the FITC/PE dot plot and the FITC histogram.

A quadrant marker is used to denote the position, in general, of the negative bead population and the FITC histogram is for viewing purposes only. All analysis will occur once all the samples have been run.

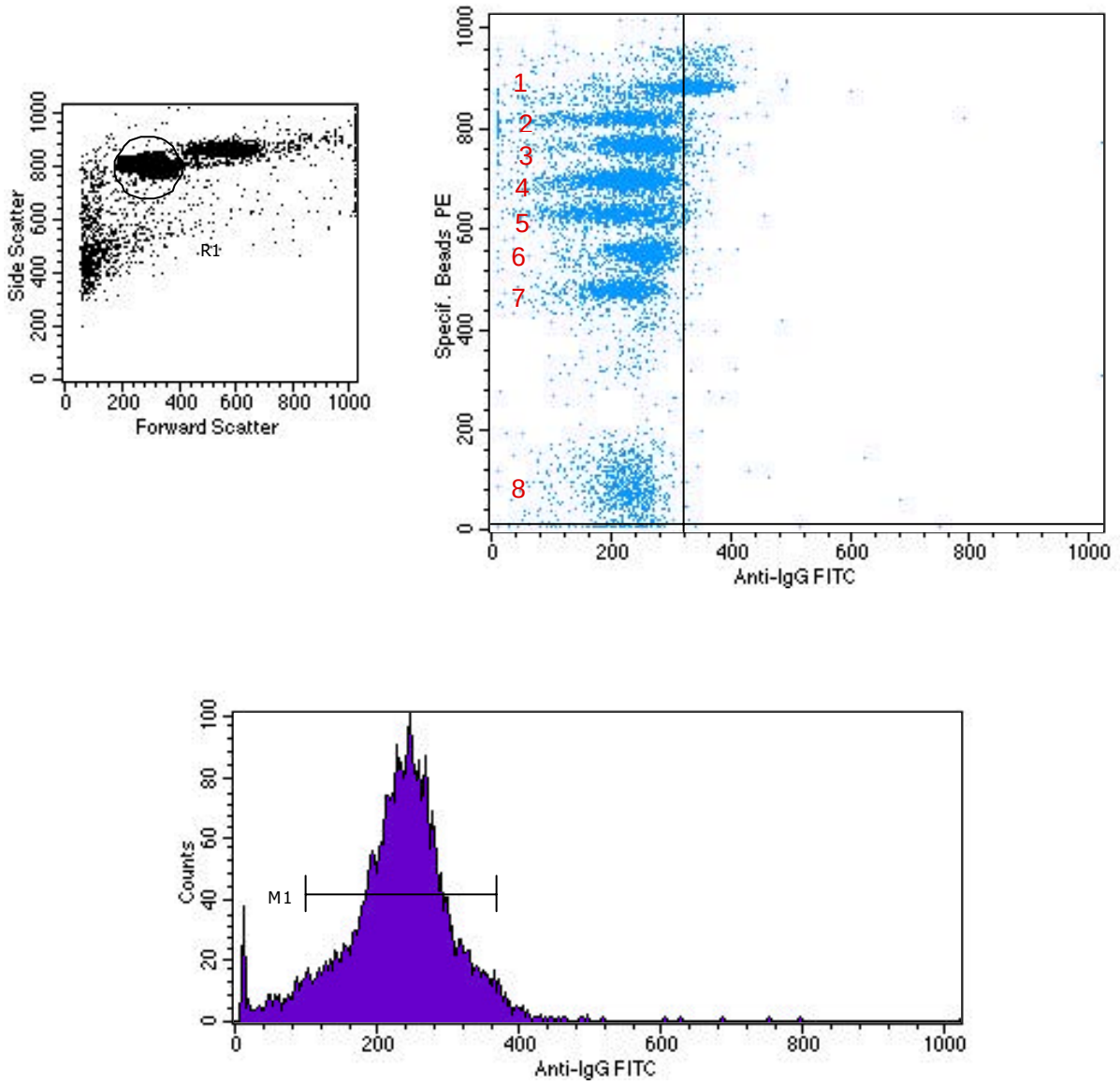


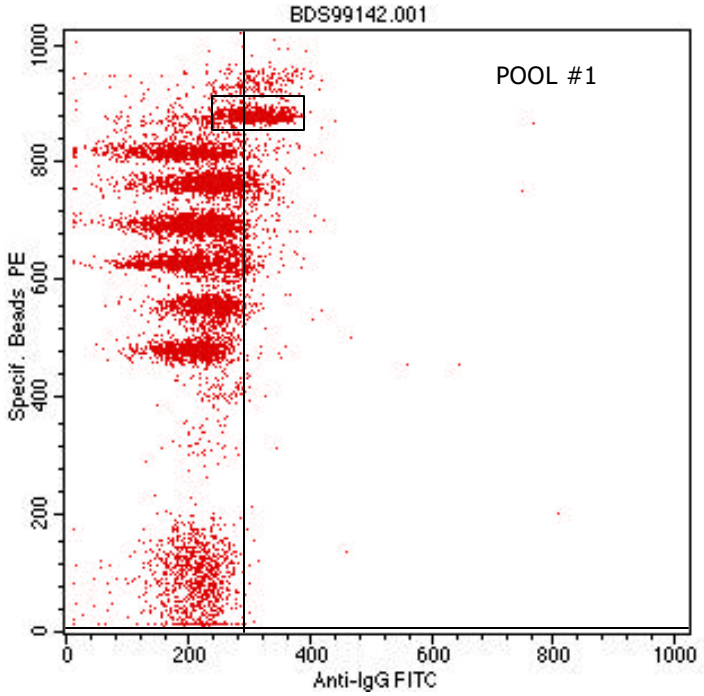
Figure 3. Sample data acquisition template.

16. Setup folder and file names for data storage.
    Save only R1 gated data.

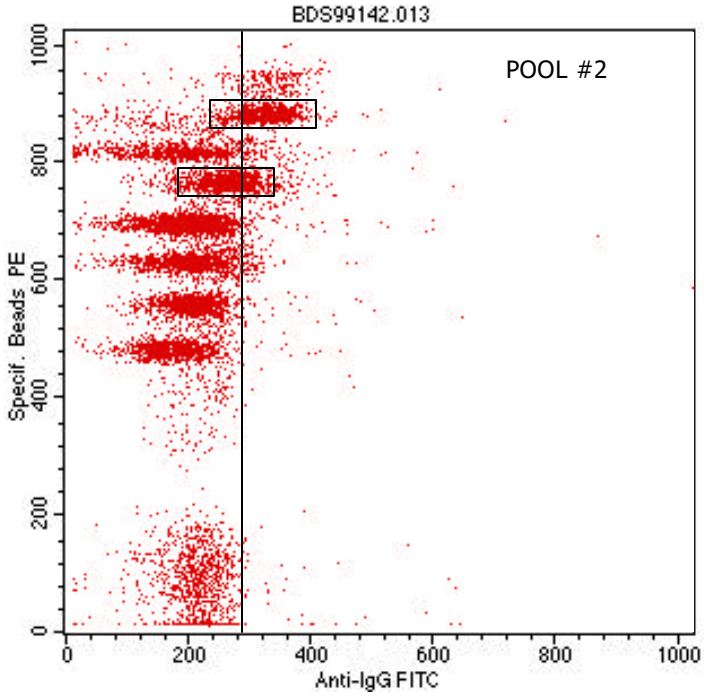
17. Instrument settings must be adjusted to bring the beads (which are small in size) on screen.
    Change the FSC detector voltage from E00 to E01.
    Change the SSC detector mode from LIN to LOG.
    Decrease the SSC detector voltage until the beads appear on the FSC/SSC dot plot.

18. Place the flow in setup and put the negative control tube on to run.
    Adjust the R1 region (see figure #3) so that it is centered around the specificity bead population. The FITC/PE dot plot displays the 8 different bead populations within the pool according to the intensity of the FL2 fluoresence. Adjustments in the FL1-FL2 compensation setting will need to be made in order to align the 8 bead populations as straight as possible along the vertical axis. This adjustment is for the majority of the group, there will be some outlyers which will be compensated for on final analysis. Be careful when adjusting the compensation. Overcompensation will cause the beads to lean toward the FL2 axis and may result in a false negative result. Undercompensation will cause the beads to lean away from the FL2 axis and may result in a false positive results.
19. When adjustments are complete remove the instrument from setup and acquire the data for the negative control sample. (10,000 events)
20. Acquire data for each sample within the first pool without changing any of the instrument settings.
    Before beginning acquisition on the subsequent pools place the instrument in set up and view the negative control sample. Verify that the negative beads are straight along the vertical axis, if not make adjustments in the FL1-FL2 compensation, and continue running the remainder of the samples within the pool.
21. Once the data for all four pools has been collected close the Cellquest acquisition template.
22. Open the Cellquest data analysis template.
    This template allows for the analysis and printout of all four pools for each sample tested.
    See Figure 4.

NHS 0706

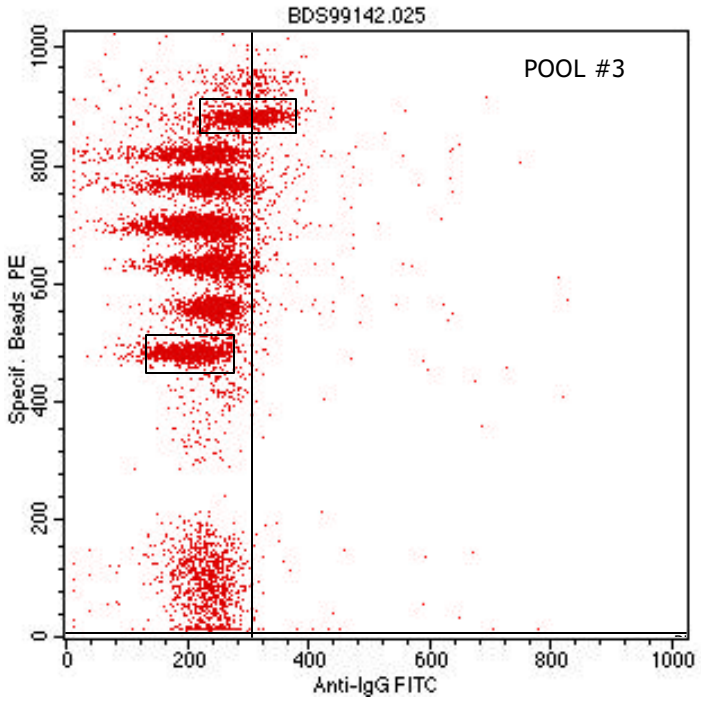


A LOCI	B LOCI	Bw LOCI
__1, 32	60,64	6,6
__1,11	8,37	4,6
__1,29	8,45	6,6
__1,XX	35,73	6
__1,80	18,50	6,6
__74,80	52,72	4,6
__23,68	37,72	4.6
__30,36	35,71	4,6

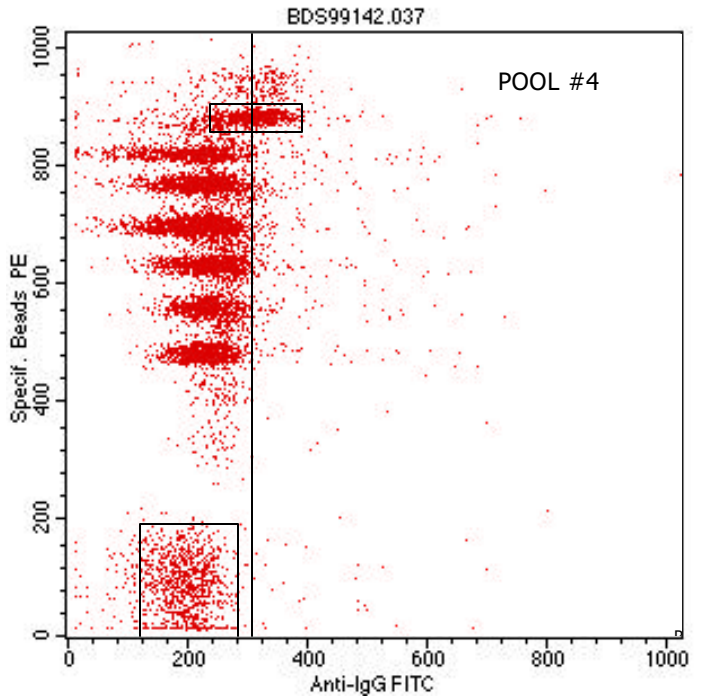


__2,3	57,65	4,6
__2,11	13,62	4,6
__2,29	7,46	6,6
__2,24	55,61	6,6
__3,68	7,65	6,6
__3,32	50,56	6,6
__3,23	18,71	6,6
__11,24	59,60	4,6

Figure 4. Sample analysis template.



A LOCI	B LOCI	Bw LOCI
__11,33	51,54	4,6
__11,23	49,52	4,4
__11,33	58,75	4,6
__2,24	54,67	6,6
__23,66	41,71	6,6
__23,33	45,63	4,6
__25,26	44,57	4,4
__32,68	44,47	4,4



__26,34	38,75	4,6
__26,74	7801,8101	6,6
__29,69	39,56	4,6
__30,31	13,71	4,6
__30,32	27,42	4,6
__11,XX	27,48	4,6
__33,36	53,63	4,4
__32,36	53,61	4,6

Fig.4. Sample analysis template continued.

23. Load the negative control data into the dot plot boxes for each pool of specificity beads.
24. See figure 4. Adjust the quadrant marker in each box so that it is just to the right of the negative bead population. Any beads that fall significantly to the left or right of these markers should have there own region box drawn around them.
    These markers denote the negative control beads. Any shift to the right of these markers signifies a positive reaction.

25. Once the negative controls have been loaded for each pool and all the quadrant markers and region boxes set no further changes are necessary and the sample results may be batch printed.
    To Batch print:
    From the Cellquest main menu bar select BATCH.
    Drag down to highlight the setup menu and make sure the following defaults are selected.
    – Plots/Statistics to process: ALL
    – Print after each file: X
    – File increment: 1
    Click on OK to save settings.
    Once the parameters for the batch run have been set start the print process by selecting BATCH from the Cellquest main menu bar and dragging to highlight RUN.

    All sample results (controls and patients) will be loaded into the boxes sequentially and a print out generated. The batch print will automatically terminate once the end of the file has been reached.
26. For each sample interpret the bead shifts as positive or negative as compared to the negative control markers and record the result next to the corresponding specificity listed.
27. Calculate percent positive and analyze for antibody specificity by pattern analysis.

Results

For each sample, both a percent PRA and antibody specificity can be determined from the flow specificity bead assay. After printing the results for a patient sample assign the individual beads as positive or negative based on there shifts relative to the negative control markers. Any significant shift of the bead population to the right of the negative control marker is positive. A significant shift is considered as >50% of the bead population moving to the right of the negative control marker. If the population remains to the left of the negative control markers then it is negative. Any population which straddles the line is considered undetermined with a positive/negative assignment withheld until specificity is analyzed. Based on antibody assignment, the undetermined bead population may then be reassessed for positive or negative interpretation.

The percent PRA may then be calculated based on the number of positive reactions divided by the panel size. Specificity analysis is based on the positive reaction pattern noted for a specific sample. Following is an example of a negative and positive control sample (Figure 5). The quadrant marker for the negative control is placed to the right of the negative bead population.

The top bead is an outlier (i.e., high background), it appears to straddle the negative control line, so an additional box is drawn around this population. After these negative markers are established they are not moved during the rest of the patient analysis since they are used as points of reference for the shift in FITC fluorescence of the beads. The positive control shows all the bead populations have shifted to the right of there negative control markers thus validating the test setup.

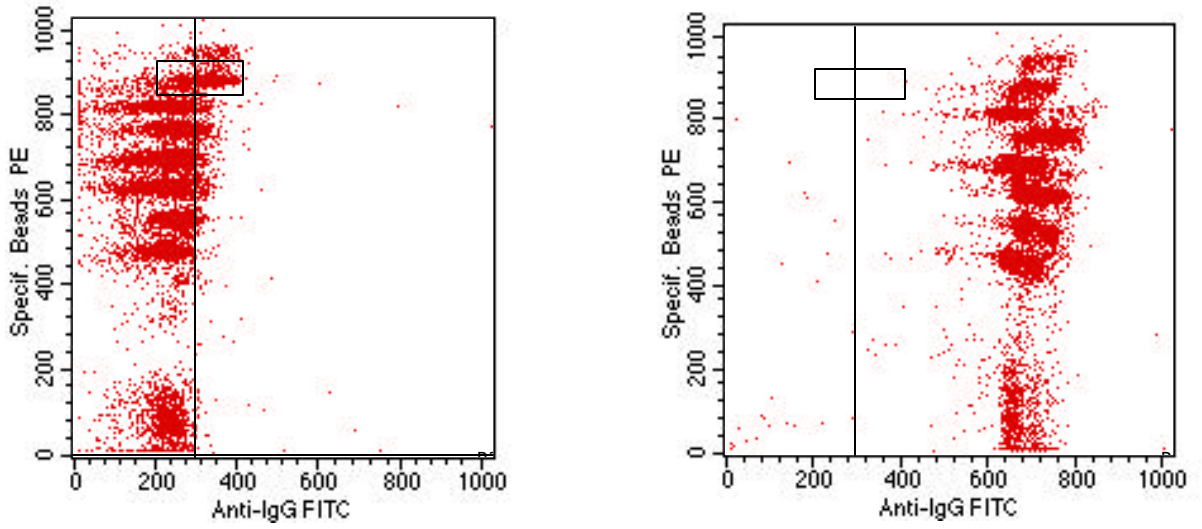
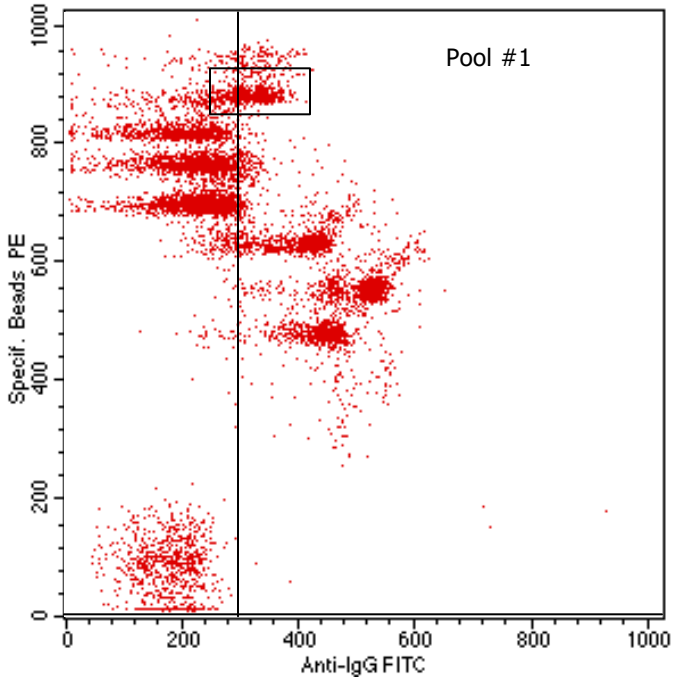


Figure 5. Example of a negative and positive control sample.

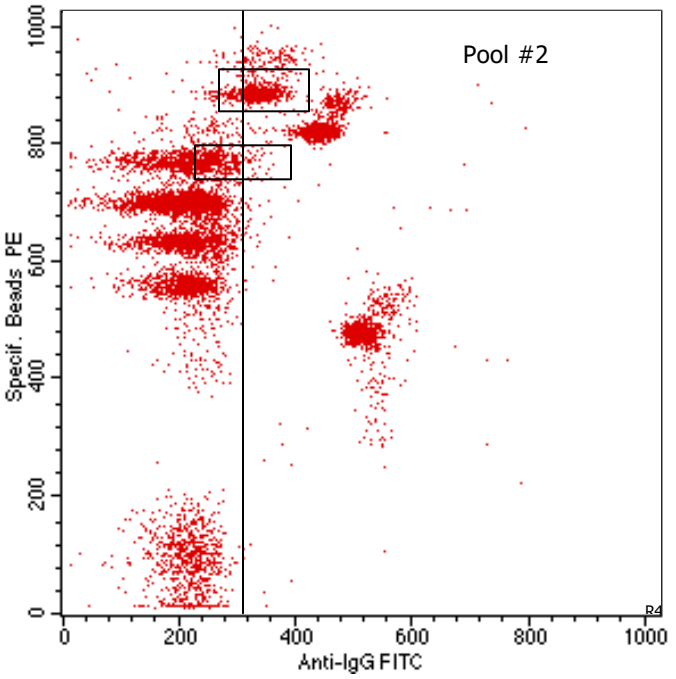
[Note that for some pools, individual beads may show a higher or lower background compared to the average of all the beads. In these instances, boxes are drawn around the individual beads to demarcate the negative region. (Figure 5, negative control)].

Once the negative control markers have been established the patient samples are then analyzed for any positive shifts in the individual bead populations. Following are some abbreviated examples for which specificity has been determined (Figure 6 and Figure 7).

ANTI DQW 2 SERA



	DRB1	DRW	DQW
-	1,4	53	5,8
-	12,18	52	4,5
-	9,10	53	5,9
-	15,18	52	4,6
+	8,17	52	2,6
+	4,7	53	2,8
+	17,103	52	2,5
-	15,9	51,53	5,9

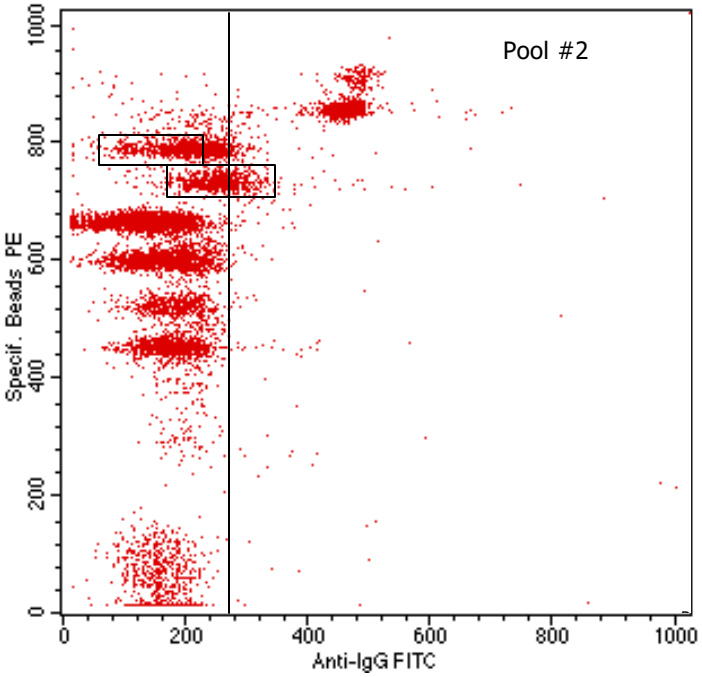


-	4,14	52,53	7,8
+	1,7	53	2,5
_	11,13	52	6,6
_	8,15	51	5,6
_	11,12	52	5,7
_	16,4	51,53	4,5
+	13,17	52	2,6
-	14,16	51,52	5

Figure 6

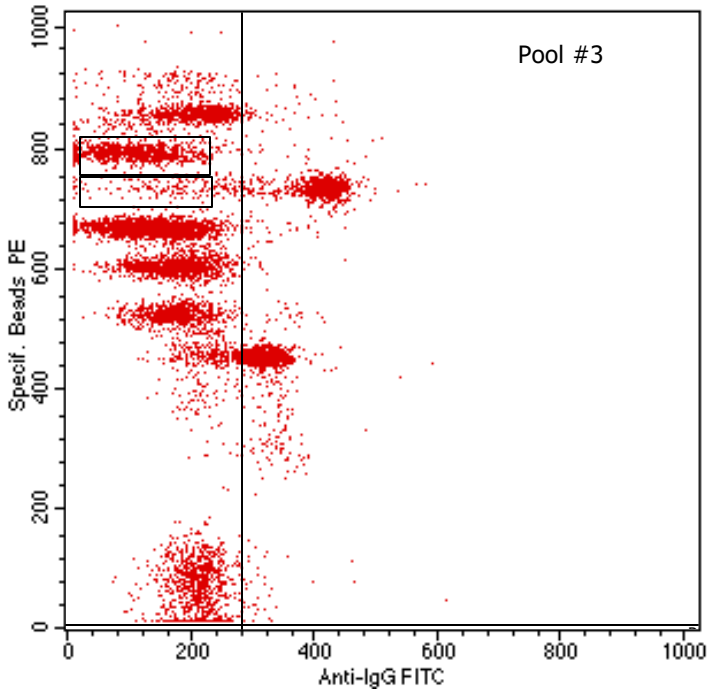
ANTI B57,B58

weak B62,B63

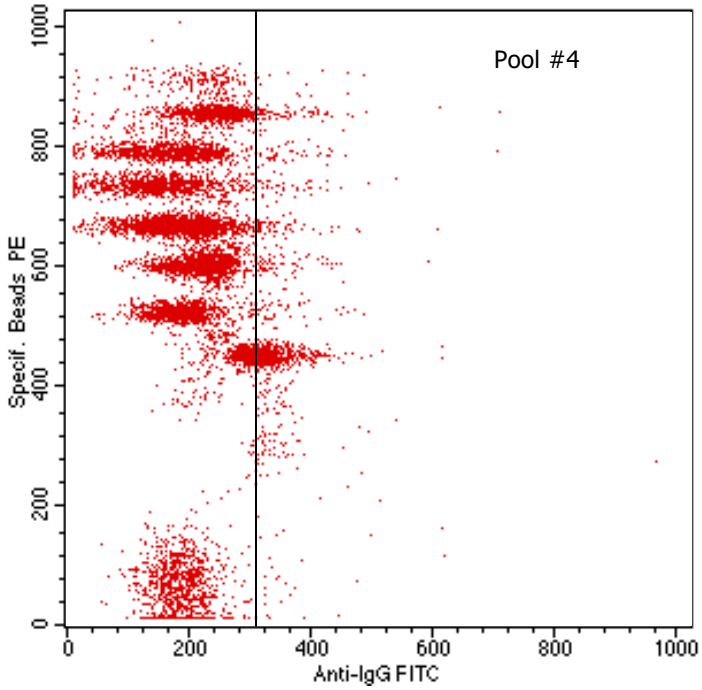


	A LOCI	B LOCI	Bw LOCI
+	2,3	57,65	4,6
+-	2,11	13,62	4,6
-	2,29	7,46	6,6
-	2,24	55,61	6,6
-	3,68	7,65	6,6
-	3,32	50,56	6,6
-	3,23	18,71	6,6
-	11,24	59,60	4,6

Figure *7*



A LOCI	B LOCI	Bw LOCI
- 11,33	51,54	4,6
- 11,23	49,52	4,4
+ 11,33	58,75	4,6
- 2,24	54,67	6,6
- 23,66	41,71	6,6
- 23,33	45,63	4,6
+ 25,26	44,57	4,4
- 32,68	44,47	4,4



- 26,34	38,75	4,6
- 26,74	7801,8101	6,6
- 29,69	39,56	4,6
- 30,31	13,71	4,6
- 30,32	27,42	4,6
- 11,XX	27,48	4,6
+- 33,36	53,63	4,4
- 32,36	53,61	4,6

Figure 7. continued.

References

1. Bray RA, Cook DJ, Gebel HM. Flow Cytometric Detection of HLA Alloantibodies Using Class I Coated Microparticles. Human Immunology 55:36, 1997.
2. Pei R, Wang C, Tarsitani S, et al: Simultaneous HLA Class I and Class II antibody screening with flow cytometry. Human Immunology 59:313-322, 1998.
3. One Lambda, Inc., FlowPRA Specific Test package insert,. One Lambda, Inc., Canoga Park CA, 1998

Flow Cytometric T and B Cell Crossmatching

Charles W. Hamrick and Lauralynn K. Lebeck

Principle

Detection of circulating donor specific HLA antibodies in the serum of potential allograft recipients is generally considered to be a contraindication to transplant. Transplant crossmatch testing detects these pre-formed anti-donor antibodies if present in the recipient serum. The use of flow cytometry to measure very low concentrations of antibodies in patient sera has proven clinically relevant.

The flow cytometric crossmatch (FCXM) is performed by incubating donor cells with recipient serum followed by the addition of fluorochrome conjugated anti-human immunoglobulin. The most widely used reagent is a fluoresceinated (FITC) goat, anti-human polyclonal immunoglobulin. If donor reactive antibodies are present in the recipient serum they will bind to the donor lymphocytes and subsequently be detected by the fluoresceinated antibody. CD3 monoclonal antibodies with fluorochrome conjugates are also added to specifically detect T cell reactivity. Alternatively CD19 or CD20 monoclonal antibodies can be employed to specifically detect the presence of B cell reactivity. Either a two-color (FL-1 green / FL-2 orange) or a three-color (FL-1 green / FL-2 orange / FL-3 red) format for flow cytometric crossmatching is highly recommended over the single color (FL-1 green) format since multi-color methods eliminate background binding due to natural killer (NK) cells and monocytes. In the "two-color" format, CD3 or CD19 conjugated to phycoerythrin (PE) is generally used. In the "three-color" format, a combination of CD20 PE and CD3 peridinin chlorophyll protein (PerCP) might be used. The stained cells are fixed with formaldehyde solution and analyzed by flow cytometry. Results are expressed as positive or negative and are based on 1) a shift in median channel fluorescence intensity (linear values) of the test serum with respect to a negative control or autologous serum or 2) an increase in the ratio of log values or Mean Equivalent Soluble Fluorochrome (MESF) units of test serum to negative control serum.

Specimen

1. Cells (viability > 80%)
   a. Whole blood preserved in acid citrate dextrose (ACD) or heparin maintained at room temperature. Specimen should be received in the lab within 24 hours of the draw time.
   b. Lymph nodes or spleen preserved in RPMI.
   c. Frozen lymphocytes may be used if they have sufficient pretest viability. DNAse treatment is recommended.
2. Sera
   a. Serum sample of potential recipient(s) from a clot (red top) tube.
   b. Serum sample of donor from a clot (red top) tube. This is used for donor autologous control.

Reagents and Supplies

1. Wash buffer PBS with 0.1% NaN_3 and 5% Fetal Calf Serum (PBS/FCS)
   a. Add 0.1 g sodium azide for each 100 ml working PBS solution prepared to yield a final concentration of 0.1% sodium azide. (PBS Azide)

   SAFETY WARNING: Sodium Azide is toxic if inhaled. A mask must be worn or a chemical fume hood must be used when handling undiluted material. Sodium Azide can be an explosive hazard when exposed to copper plumbing. Flush with large quantities of water when disposing of solutions in sink drains.
   b. Thaw and add one 5 ml aliquot of fetal calf serum for each 100 ml of working PBS Azide solution (see part a. above).
2. Control Sera
   a. *Negative control serum* – Single donor lot of normal human serum (NHS) yielding appropriate values when tested by flow cytometric techniques. Sera should be from a male, blood group AB donor and must not be cytotoxic. Can use purchased Human AB serum, or an in-house preparation and stored at -70°C.

   Note: In a good negative control, the fluorescence shift of a suspension of this serum and cells should be very similar to a suspension of the same cells in phosphate buffered saline. It is wise to invest time at the beginning to survey a number of sera until you find one or several with a low background. The lower your background is, the more sensitive the test.
   b. *Positive control serum* – Pooled positive PRA patient sera. Positive patient sera should be HLA specific and the pool must include a full range of HLA antigen specificities. Each positive pool lot is used at a dilution that yields a moderate to low reactivity to ensure maintenance of appropriate positive cut-off with each test run. Store at -70° C.

Note: Some laboratories run the pooled positive control neat and at a dilution. The dilution is usually set above the upper threshold of the negative control, i.e., a "borderline" or weakly positive reaction. This is to ensure consistency in determining the lower limits of a positive test.

3. Fluorescence-conjugated antibodies
   a. Fluorescein-conjugated secondary reagent (FITC conjugated goat [F(ab)'$_2$] anti-human IgG (Fc specific) *Cappel Cat# 55184* or *Jackson Labs Cat # 109-016-098*). Expiration date is one year from date reconstituted. Once reconstituted, it is stored in 20 µl aliquots at -70° C.
   b. Anti-human T-cell reagent (Becton Dickinson phycoerythrin-conjugated CD3-*PE cat# 3347347* or peridinin chlorophyll protein-conjugated CD3-PerCP *cat # 3347344*) and stored at 4°C.
   c. Anti-human B-cell reagent (Becton Dickinson phycoerythrin-conjugated CD19-PE *cat# 349209* or CD20-PE *cat# 3347677*); stored at 4°C.

   Note: FITC, PE and PerCP are light sensitive so keep in the dark.
4. 1% Formaldehyde solution – Stock solution is 10% formaldehyde (Polysciences). Make a 1:10 dilution of the stock in PBS Azide, pH to 7.2 + 0.2, and store in dark at 4°C. The reagent is stable for one month.
   SAFETY WARNING: Formaldehyde is a highly toxic carcinogen. Use personal protective equipment such as gloves, lab coat and mask when handling. Use of a fume hood is also recommended.
5. 12 x 75 mm Polystyrene Falcon tubes (Fisher Cat# 352008).

Instrumentation

1. Vacuum aspiration system
2. Channel Alarm Timer
3. Vortex Genie Mixer
4. Airfuge with micro-ultracentrifuge tubes and protective caps
5. Refrigerated centrifuge
6. Flow Cytometer
7. Hemacytometer – Coverslips / Microscope
8. Adjustable Pipettes: 10 µl to 100 µl
9. Repeating dispensers for delivering volumes from 500 µl to 5 ml
10. Test tube rack
11. Ice bath for test tube rack

Calibration

Instruments such as repeating dispensers, centrifuges, timers, or temperature recording systems must be calibrated periodically to ensure that delivered volumes, centrifugation speed and time / temperature are consistent and accurate. Proper instrument setup and performance on the day of testing is critical for obtaining accurate and reliable results. The flow cytometer will have vendor specific standards such as beads that should be included each time the instrument is operated. Minimally, instrument settings such as photomultiplier tube (PMT) voltages, fluorescence compensation and sensitivity must be verified and recorded.

Quality Control

Due to the exquisite sensitivity afforded by this methodology, the flow cytometric crossmatch is particularly dependent on rigorous quality control measures for reagents and equipment including:

1. All cell concentrations, serum dilutions, and volumes must be exact and accurate.
2. Negative control serum should be screened against a panel of cells by the flow cytometric crossmatch technique to select a reagent with low background staining.
   a. Every time a new lot of NHS is begun, a new cutoff value needs to be determined. This should be done using a statistically significant sampling of flow crossmatch results on normal cells.
   b. Most laboratories use a cut-off equal to two times the standard deviation of the mean of the median channel values obtained when evaluating the NHS versus 30 – 50 donor cells plus the actual value of the NHS in each assay for patient testing.
      Note: some labs use +2.5 SD or +3 SD to define their cut-off value.
3. Positive control serum usually is a pool of highly reactive patient sera. Inclusion of anti-Bw4 or Bw6 would guarantee reactivity with most donor T cells. Adding anti-DR52 or DR53 for B cells might be useful, however all B cells tend to react nicely with Class I antibody pools. Each positive pool must be titrated to define the appropriate dilution to use in the flow cytometric assay. A minimally reactive positive pool or dilution would be most appropriate.
4. Determination of the correct working dilution of the goat anti-human IgG FITC reagent via titration is necessary for maximal sensitivity in the flow cytometric crossmatch. The titer of antibody that gives the lowest NHS median peak channel (average of replicates) and the highest positive median channel is chosen. When any changes are made to this reagent such as a different dilution or new lot of reagent, a new cutoff value must be established.

5. The goat anti-human reagent must also be shown to lack cross-reactivity with mouse or other species immunoglobulins. Testing of normal mouse serum and bovine serum are minimally required since the monoclonal antibodies used to detect T cells and/or B cells are mouse antibodies and fetal calf serum is used in the wash buffer.
6. Each new lot of CD3 and CD19 reagents must be tested prior to use to show that they stain the proper sub-population of lymphocytes. Testing in parallel with the current lot is the easiest method to evaluate new reagents.

Procedure

1. Isolation of mononuclear cells from anticoagulated peripheral blood, lymph node or spleen should follow routine protocols of the laboratory. Ficoll separation is the method of choice.
   Note: Do not use a cell preparation that has been treated with Lympho-Kwik™ or Percoll in that spurious results (usually false negative) are obtained.
2. Adjust cell concentration to 1.6 x 10^7 cells/ml in PBS Azide. Check cells for purity and viability. Starting viability must be greater than 80%. Overall purity of the cell preparation should be <10% contaminating cells such as platelets, RBCs, and granulocytes.
3. All serum samples to be tested, including positive and negative control sera, should be airfuged prior to use to remove aggregated immunoglobulin and immune complexes after freezing and thawing. If not removed, these aggregates may produce non-specific background staining, particularly on B cells.
4. Label 12 x 75 Falcon tubes for each donor cell:
   a. Normal serum control in duplicate
   b. Buffer control (background autofluorescence, no primary antibody)
   c. Donor autologous control (if serum is available)
   d. Positive serum controls (strong and minimal); dilute in PBS Azide
   e. Patient test serum in duplicate
   Note: many laboratories do not perform testing in duplicate, however this is a very good indicator of technique and is recommended if adequate donor cells are available.
5. Aliquot 30 µl (500,000 cells) of cell suspension per tube. Mix the cell preparation well before adding the cells to ensure consistency from tube to tube.
6. Add 30 µl of the appropriate sera to each tube and vortex to ensure proper mixing of serum and cells.
7. Incubate for 20-30 minutes at room temperature. (Some laboratories perform this incubation at 4° C for simplicity since all subsequent testing is performed at 4° C.)
8. Add 3 ml **cold** PBS/FCS to each tube.
9. Centrifuge 5 minutes at 500 x g, 4° C.
10. Aspirate supernatant. Resuspend pellet. Repeat wash 2 more times.
    Note: When aspirating do not aspirate cell pellet. Residual fluid volume should be < 30 µl.
11. Add 100 µl of diluted anti-human IgG FITC to each tube and vortex. Check titer and proper dilution of ALL reagents prior to use.
12. Addition of PE and/or PerCP fluorochromes
    a. *TWO-COLOR METHOD*. Add 20 µl of CD3 PE (or alternatively CD19 PE) to each tube and vortex.
    b. *THREE-COLOR METHOD*. Add 20 µl of CD3 PerCP and 20 µl CD19 or CD20 PE to each tube and vortex.
13. Incubate for 30 minutes on ice in the dark
14. Wash 2 times with 3 ml cold PBS/FCS and aspirate.
15. While vortexing, add 500 µl of 1.0% formaldehyde to each tube.
16. Samples can be immediately analyzed or held at 4°C in the dark for up to 7 days.
17. Flow cytometric acquisition / analysis should be performed on a minimum of 5000 – 15000 "gated" lymphocytes.
    a. These collection criteria should yield >1000 T lymphocytes and at least 1000 B lymphocytes for subsequent analyses.
    b. Pre-defined templates including cytometer settings are highly recommended for clinical use and can be easily defined for any of the commercial flow cytometers on the market. These will greatly increase consistency between cytometer operators within your laboratory.

Results

An example of a negative flow cytometric crossmatch with corresponding dot plots and histograms is shown in Figure 1.

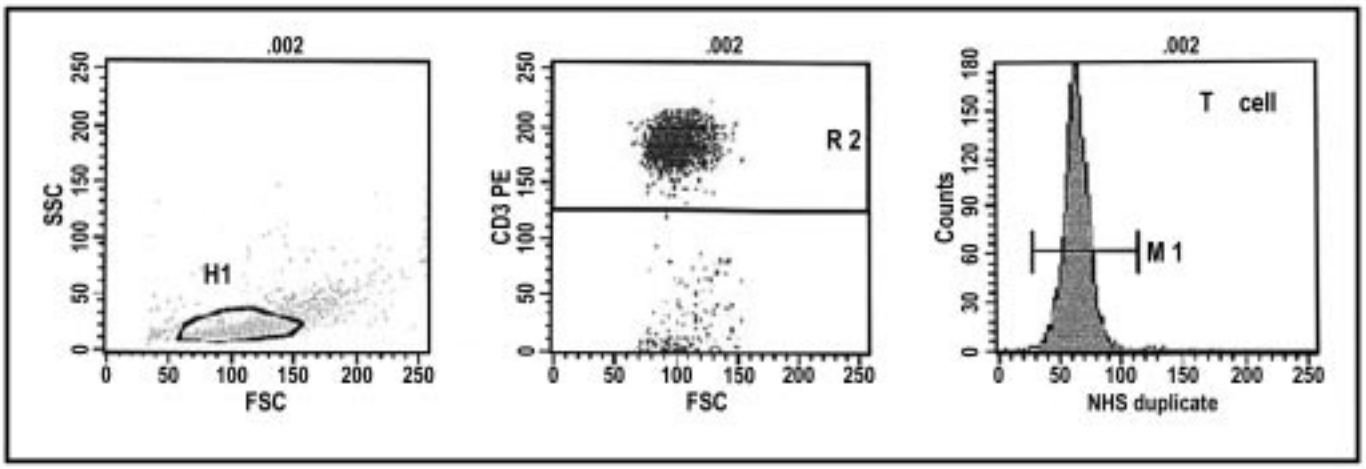


Figure 1: Two-color T cell flow cytometric crossmatch (256 linear scale).
Left dot plot displays Forward scatter (FSC) versus Side scatter (SSC) of mononuclear cell preparation. R1 gate = lymphocyte population.
Center dot plot displays FSC versus FL-2 (CD3 PE) of R1 lymphocyte gate. R2 gate = T cells
Right histogram displays FL-1 (anti-human IgG FITC) fluorescence of T cells (R2 gated events).

Calculations

1. Transcribe printed results of flow histograms to flow crossmatch worksheet.
2. Calculate average median channel for duplicate NHS and patient tubes.
3. Compare test sample to NHS
   a. *Calculate Median Channel Shift (MCS).* Useful when collecting data on a linear scale of either 256 or 1024 channels. Subtract the value of NHS from the patient seum value.
   b. *Calculate the Ratio.* Useful when collecting data on a log scale or when displaying values as MESF units.
4. Criteria need to be specifically defined within your laboratory for interpretation of flow values. The following table lists approximate positive cut-off values for general considerations.

	MCS 256 scale	**MCS** 1024 scale	**Ratio**
T Cell	> 10 – 15	> 40 – 60	> 1.0
B Cell	> 25 – 30	> 100 – 120	> 1.2

5. Quality control criteria are also important when evaluating flow cytometric crossmatch data. The values listed below are to be used as guidelines for interpretation and troubleshooting. If a sample should fall outside of these ranges it does not necessarily invalidate the test but should indicate the need for a closer evaluation of the results and review by the director.
   a. Duplicate tubes should agree within a defined range (e.g., ± 5 channels on a 256 scale or within 30 channels for T cells on a 1024 scale).
   b. NHS values and Positive control sera should fall within defined ranges. If the positive control value for a given assay is significantly greater than the defined value it is not necessarily a cause for concern or indication for repeat of the test. However, if the value is significantly below the range, repeating the test is appropriate to validate crossmatch negative patient sera.
   c. MCS or ratio between NHS and Positive control must have a defined minimal value with acceptable ranges.
   d. Greater than 90% of the cells should fall within the analysis gate, otherwise the cell preparation may have been inappropriate.
   e. Percent CD3 and/or CD19 between tubes must be within a 10% range, preferably 5%. This is an indication of pipetting and washing/aspiration technique.

Procedure Notes

1. The described procedure makes a 1:2 dilution of test serum (30 µl cell suspension plus 30 µl serum). Many laboratories prefer to make a dry cell pellet at the first step and therefore do not make any dilution of the serum samples. This alternative yields a more sensitive test however the possibility of losing cells during the initial aspiration needs to be carefully controlled.
2. Insufficient washing may result in false negative flow crossmatch tests. Following the primary incubation it is important to perform the number of wash steps specified in the procedure. Laboratories have modified the staining methodology by adapting it to microtiterplates or other smaller test tubes (6 x 50 mm, *Evergreen Scientific*), where the wash steps become increasingly critical since smaller volumes of wash buffer are utilized.
3. Fluctuations in the serum to cell ratio can significantly alter the crossmatch results. A lower number of cells may be used routinely such as 250,000 / tube however the serum volume must be appropriately altered and new cut-off values defined. The biggest potential for error lies in performing accurate cell counts. Excess cell numbers can produce false negative results.
4. An incorrect dilution of the FITC anti-human IgG reagent could result in a shift of the controls and patient values out of the established range. Check titer and proper dilution of all reagents prior to use.

Limitations of Procedure

The exquisite sensitivity of flow cytometric methods may yield so-called “false positive” results, in that HLA antibodies may not be the cause of the positive crossmatch. Prospective flow cytometry crossmatch testing may not be indicated in “unsensitized” first transplant candidates. In sensitized patients, the flow crossmatch unquestionably provides valuable information for selecting an appropriate recipient/donor pair.

References

1. Garovoy MR, Rheinschmit MA, Bigos M, et.al., Flow cytometry analysis: a high technology crossmatch technique facilitating transplantation. Transplantation Proceeding 15:1939, 1983.
2. Cook DJ, Terasaki PI, Iwaki Y, et.al., An approach to reducing early kidney transplant failure by flow cytometry crossmatching. Clinical Transplantation 1:253, 1987.
3. Bray RA, Lebeck LK, Gebel HM, The flow cytometric crossmatch: Dual-color analysis of T and B cells. Transplantation 48: 834, 1989.
4. Bray RA, Flow cytometry in the transplant laboratory. Annls. N.Y. Acad. Sci. 677: 138, 1993.
5. Bryan CF, Baier KA, Nelson PW, et.al., Long-term graft survival is improved in cadaveric renal retransplantation by flow cytometric crossmatching. Transplantation 66: 1827, 1998.

Phenotyping by Immunofluorescence

Mary L. Duenzl, Linda Stempora, and Robert A. Bray

Principle / Purpose

Individual cells can be distinguished by a set of characteristic markers or antigens. These markers are generally glycoproteins that may be expressed either on the cell surface, on an intracellular structure, or in the cytoplasm. These markers may be restricted to a particular cell type or lineage, or may be distributed over a wide range of different cell types or lineages. While many of these antigens are well characterized, many do not have a biological reported function. Nonetheless, identifying and cataloguing the constellation of markers displayed by an individual cell or population of cells can be of significant value in both clinical and research setting. Fluorescence immunophenotyping has been the most common approach for identifying cell surface (and intracellular) antigens. Immunophenotyping of cells can be performed either on cells fixed to a slide or on cells in suspension. This chapter will present the methods used to prepare and stain cells for subsequent analysis, by either flow cytometry or fluorescence microscopy, in suspension.

Fluorescence immunophenotyping utilizes known antibodies (polyclonal or monoclonal) that are directed against specific cell markers. As described in the chapter "Basic Principles and Quality Assurance of Immunofluorescence and Flow Cytometry" (VI.A.1), both direct and indirect immunofluorescence techniques can be performed. The direct technique utilizes antibodies that have been directly conjugated with a fluorochrome (fluoroscein [FITC] or phycoerythrin [PE]). The isolated cells are incubated with the antibody reagent, washed, fixed, and then analyzed either by flow cytometry or fluorescence microscopy. The indirect technique requires an additional step since the marker-specific primary antibody is not conjugated with a fluorochrome. The primary incubation is performed with the unconjugated marker-specific antibody, and following a wash step, the cells are incubated with a fluorochrome conjugated secondary antibody specific for the primary antibody. Following the second staining incubation, the cells are washed, fixed and then analyzed by either flow cytometry or fluorescence microscopy.

In general, the direct staining technique is the most widely used method, especially in the clinical laboratory setting, and is the technique best suited for multi-color flow cytometry. However, due to the availability of some antibody reagents, indirect techniques may be the only choice. Some applications, such as the flow cytometric crossmatch, utilize a combination of both techniques.

As in all laboratory practice, appropriate safety precautions must be observed. Personal protective equipment, such as gloves and a lab coat, are required. A laminar flow biohazard hood is highly recommended when handling any blood or body fluids.

Specimen

Peripheral Blood Specimens

Peripheral blood may be collected in sodium heparin, EDTA, or acid citrate dextrose (ACD) anticoagulants. Specimens are stored at room temperature and transported to the laboratory as soon as possible. If absolute cell counts are required, blood should be collected in the same anticoagulant as required for the cell count, usually EDTA. Immunophenotyping may be performed up to 30 hours after collection, but additional time restraints may be in place for cell counts, i.e., a CBC must be done within a shorter time limit.

Mononuclear cells may be isolated from blood by using a density gradient separation media such as ficoll-hypaque. The isolated mononuclear cells may be maintained for approximately 48 hours when stored in tissue culture media at 4° C. However, it is important that storage parameters be verified within each laboratory.

Interfering Substances: Anti-lymphocyte globulin (ALG or ATGAM) or therapeutic doses of OKT3 or OKT4 may interfere with cell marker analysis by producing a high degree of background staining. Additionally, these therapies may produce leukopenia and severe lymphopenia. The laboratory should be notified if the patient is receiving such therapy. Also, the use of long-term, high-dose steroid therapy may show diminished expression of cell surface markers.

Bone Marrow Specimens

Bone marrow aspirates are usually collected syringes containing either EDTA or sodium heparin. Three to five milliliters are usually required for evaluation and may be sent to the laboratory in a syringe (needle removed) or placed in a separate tube. Specimens should be maintained at room temperature and, again, immediately transported to the laboratory.

Note: Depending upon the methods used to obtain the marrow aspirate, peripheral blood contamination of the specimen may be quite significant.

Tissue Specimens

Only **FRESH, UNFIXED** tissue specimens can be processed for immunophenotyping. Fixation can destroy many antigenic determinants. The laboratory should be familiar with the fixation stability of the determinants being tested.

Solid tissue samples and fine needle aspirates (FNA) should be submitted to the laboratory in tissue culture media such as RPMI 1640 or a balanced salt solution such as Hank's Balanced Salt Solution, maintained at room temperature, and transported quickly. If transportation of solid tissue is delayed by several hours, it should be minced into several pieces before placing in the media.

Cells are recovered from solid tissue by manual disassociation. Thus, care must be taken to maintain viability while recovering as many cells as possible from the tissue. Isolated cells may be maintained for approximately 48 hours when stored in tissue culture media at 4° C. Again, it is important that storage parameters be verified within each laboratory.

Cultured Cells

Cells grown in tissue culture are quite acceptable for immunophenotyping. Cells should be removed from the culture flask or plate as a single cell suspension, washed to remove tissue culture media and debris. Cell concentration should be adjusted and viability determined prior to staining.

Cell Concentration and Viability

Cell concentration must be adjusted for optimum staining results. Manufacturers provide guidelines for each antibody reagents, but each lab should determine optimum concentration. For normal white blood cell counts (4000 – 10,000 cells/(L); 100 µl is the recommended volume. Most reagents are titered for staining up to 1.0 x 10^6 cells. Cell counts may also need to be adjusted to allow analysis of a rare subpopulation of cells. For example, patients undergoing OKT3 therapy will have marked lymphopenia so counts will have to be adjusted if lymphocytes are to be analyzed. With experience and when necessary, as few as 0.5 x 10^5 cells can be stained.

In general: use no more than 1.0 x 10^6 cells and no less than 0.2 x 10^6 cells per tube when using the 12 x 75 mm size. The use of smaller (6 x 50 mm) tubes permits staining of as few as 40,000 cells per tube. The smaller size minimizes cell loss during washes.

Cells from peripheral blood and bone marrow stored at room temperature are acceptably viable for up to 30 hours after collection. Cells in tissue culture media stored at 2-8° C are acceptable at 48 hours. For optimal results, pre-test viability should be 80% or higher. Viable stains such as propidium iodide (PI) or 7-aminoactinomycin-D (7-AAD) can be used to exclude dead cells from flow cytometric analysis. Trypan blue is the most common light microscopic stain used for determining viability prior to fluorescent staining.

Reagents and Supplies

Fetal Calf Serum (FCS)

Used as a serum supplement for the wash solution and Complete Media
DO NOT MIX LOT NUMBERS

Preparation and Storage: 500 ml bottles of FCS may be stored at -75° C prior to aliquoting. Bottles must be labeled with both the date of receipt and date of expiration. Thaw completely at 4° C (usually overnight) prior to heat-inactivation (H.I.). (Keep the bottle in a plastic bag while thawing to prevent leaking, should the bottle have cracked during shipment.) Heat inactivate in a 56° C water bath for 40 minutes with periodic mixing. Usually, H.I. requires 30 minutes; the additional time is to allow the entire 500 ml bottle to reach 56° C. Aliquot using sterile techniques into sterile polypropylene tubes. Aliquots of 5, 25, and 50 ml are very useful. Label all aliquot tubes with H.I. FCS, the lot number, amount of aliquot, date aliquoted, and expiration date. Store aliquotes frozen at -70° C.

Note: Wash solution and/or complete media must be parallel tested prior to use.

1X Phosphate Buffered Saline (PBS), pH 7.4 + 0.2
Used to prepare other reagents
Preparation and Storage:
Dulbecco's Phosphate Buffered Saline, without Ca^{++} and without Mg^{++} (Gibco #310-419AJ)
or
2.56 g sodium phosphate, monobasic ($NaH_2PO_4 \cdot H_2O$)
97.60 g sodium chloride (NaCl)
EITHER: 11.93 g sodium phosphate dibasic (Na_2HPO_4)
OR: 22.48 g sodium phosphate dibasic anhydrous ($Na_2HPO_4 \cdot H_2O$)
In 1 liter volumetric flask, add reagents and QS to 1 liter with deionized water. This will be a 10X solution. Adjust pH to 7.4 + 0.2 using 10N NaOH or 10N HCl. Transfer to a carboy and add 9 liters of deionized water for a total volume of 10 liters of a 1X solution.
PBS may be sterilized by filtration through a 0.2µm filter. Label carboys and bottles with lot number, amount, date made, and expiration date. Store unopened (sterile) bottles at room temperature and opened bottles at 2-8° C.

2X PBS
Used for making 2% paraformaldehyde
Preparation and Storage: In a glass bottle, dissolve contents of a 9.6 g package (for 1 liter) of Dulbecco's Phosphate Buffered Saline Powder (Gibco #480-1300EB) in 500 ml of distilled water with constant stirring. Label bottle with 2X PBS, lot number, date made, and the expiration date (six months from date made). Store at 2-8° C.

2% Paraformaldehyde (2% PFA)
Used as fixative for immunofluorescence evaluation
Preparation and Storage:

CAUTION: EXTRA SAFETY MEASURES REQUIRED. Chemical fume hood must be used. When heated, Paraformaldehyde gives off toxic formalin fumes.

Under a chemical fume hood, heat 50 ml of distilled water to 50-60°C in a 100 or 200 ml beaker. Add 2 g PFA powder (Sigma #P6148) and using a stirring bar, allow to dissolve for 15 minutes. Cover with foil to prevent evaporation. Remove from heat. Add a few drops of 1N NaOH to clear the solution. If solution has evaporated to less than 50 ml, QS back to volume with distilled water. Add 50 ml of COLD 2X PBS. The sudden reduction in temperature will diminish the formalin fumes. Remove from hood and adjust pH to 7.4 ± 0.1 with 1N NaOH (back titrate with 1N HCl). Store in glass bottle 2-8° C protected from light. Label with reagent name, lot number, date made, and expiration date (2 weeks from date made).

Sodium Azide NaN_3; (Sigma #S2002)
Used in other reagents
Preparation and Storage: Store with desiccation – refer to manufacturer's expiration date.

CAUTION: Sodium Azide and reagents containing sodium azide may react with lead and copper plumbing to form explosive metal azides. Flush drain with large amounts for water to prevent azide accumulations. Refer to Material Safety Data Sheet (MSDS) provided with this reagent for other precautions.

PBS with 0.1% Sodium Azide, pH 7.4 ± 0.1
Used in wash solution and to dilute antibody reagents
Preparation and Storage: Add 0.5 g of sodium azide to 500 ml of 1X PBS. Filter sterilize and store in a sterile container. Label with reagent name, date made, and expiration date (3 months from date made). Add "contains sodium azide" precaution statement. Store at 2-8° C.

PBS with 0.1% Sodium Azide and 1% FCS, pH 7.4 ± 0.2 (wash solution)
Used as wash solution in staining procedure
Preparation and Storage: Add 5 ml of thawed FCS to 495 ml of sterile PBS with 0.1% sodium azide. Label with reagent, date made, expiration (3 months from date made). Add "contains sodium azide" precaution statement. Store at 2-8° C. Note: Turbidity is a sign of deterioration and reagent should be discarded.

Whole Blood Lysing Solutions
Used to lyse red blood cells while maintaining white blood cell integrity
Preparation and Storage: Refer to manufacturer's directions.
Several whole blood lysing reagents are available commercially. Each lab must determine the best type of lysing reagent for each application and instrument as technical differences between reagents will affect how cells appear on the flow cytometer. Most reagents fix the white cells as well as lyse the red blood cells. However, with some methods, such as ammonium chloride lysis, fixation is a separate step that may be omitted if desired.

Antibody Reagents
Preparation and Storage: Refer to manufacturers' directions. In general, most reagents should be handled aseptically, protected from light and stored at 2-8° C.

CAUTION: Most of the antibody reagents contain Sodium Azide which may react with lead and copper plumbing to form explosive metal azides. Flush drains with large amounts of water to prevent azide accumulation. Refer to Material Safety Data Sheet (MSDS) provided with this reagent for other precautions.

Obviously, there are far too many commercially available antibodies to mention here. Previous chapters list a few of the more common reagents and the reader is referred to manufacturers' catalogs and other directories such as Linscott's directory for a much more complete listing of the vast array of available antibodies and fluorescent conjugates for immunophenotyping.

Every laboratory should test each antibody reagent according to appropriate quality control standards. This may range from extensive testing and titering of a new reagent to a limited parallel testing of a new lot with the old. In addition, the lab should be knowledgeable of the physical properties of the antibody itself such as:

protein concentration	usually expressed in µg/ml
isotype and subclass	if monoclonal
species derived from	if polyclonal
degree of purity	

The last property is particularly important for indirect staining and specificity. Purity is not only important for secondary antibodies in indirect staining but may also be important in the reaction of monoclonal antibodies as well. For example, two monoclonal reagents may recognize the same Cluster Designation but not necessarily the same epitope, causing differences in the observed staining pattern.

Instrumentation/Special Equipment

12x75 mm disposable glass or plastic culture tubes
6x50 mm disposable glass or plastic culture tubes*
adjustable Eppendorf pipettes for volumes from 10 µl to 100 µl
disposable tips for Eppendorf pipettes
Eppendorf repeater pipette and combi-tips (syringe type tips)
tube racks: either plastic test tube racks in a tray filled with ice or special staining racks fitted with ice tray on bottom
crushed ice
caps for 12x75 mm plastic culture tubes
Parafilm
glass Pasteur pipettes
3 channel timer
Vortex mixer
Centrifuge: refrigerated, swinging-bucket rotor, speed adjustable (Sorvall RT 6000B)
Vacuum aspiration flask apparatus: consists of side-arm Erlenmyer flask connected to vacuum source with heavy rubber tubing on the side-arm. One-holed rubber stopper has clear plastic tubing attached to fine-tipped glass Pasteur pipette. Used for aspirating supernatants during cells washes.

* Special, fine-tipped Pasteur pipettes are required for aspirating from the 6x50 mm tubes. These pipettes can be prepared by heating the tip of a 9" glass Pasteur pipette in a Bunsen burner flame (or Bac T incinerator) then grasping the end of the pipette with forceps and gently pulling to stretch the tip to a fine point. Immediately remove from flame and allow to cool. Break off end of tip where bore is approximately 1 mm in diameter. The result is a fine tipped glass pipette that can reach to the bottom of the smallest diameter tube.

Quality Control

Cell Controls

Cells known to be positive for selected antigens should be run to verify the proper performance of reagents during each day of use. Normal cells, cultured cells, or abnormal cells can be used, with preparations of normal human lymphocytes the appropriate choice for many antigens. Frozen/thawed cells should be utilized whenever possible to ensure staining consistency from day to day. Several stabilized whole blood products for use as a daily control are commercially available.

Reagent Controls

A negative reagent control should be run for each cell preparation and should be matched as to species, isotype and subclass of the specific antibody reagents. Negative controls should be run for each fluorochrome used and at the same fluorochrome protein ratio.

Instrument Quality Control

The flow cytometer must be monitored each day used to ensure proper alignment and sensitivity. Each instrument has specific guidelines and requirements.

Stained Sample Stability

Stained cells, whether analyzed directly or fixed prior to analysis, must be analyzed within a time period demonstrated by the laboratory to avoid any significant loss of any cell subpopulation or total cell number. Control samples must be analyzed within the same time period.

Staining Procedures

Direct Staining Technique

1. Pipette appropriate amount of well-mixed sample into labeled 12x75 mm plastic tube.
2. Pipette appropriate amount of specific antibody or control antibody reagent. Volume will vary by manufacturer and titer.
3. Cap tubes and vortex.
4. Incubate 15 minutes at room temperature in the dark. Once fluorescent reagents have been added, protect the tubes from light to prevent fading.
5. Lysis of red blood cells (if necessary): this will vary with manufacturer and reagent, but usually reagent is added, tubes are re-capped, vortex thoroughly, and tubes are incubated 10 to 15 minutes at room temperature protected from light.
6. Centrifuge tubes 400 g for 5 minutes.
7. Aspirate and discard supernatant using vacuum apparatus. Avoid dislodging cell pellet.
8. Pipette 1 ml Wash Buffer to each tube, re-cap, and vortex.
9. Centrifuge tubes at 400 g for 5 minutes.
10. Repeat steps 7, 8, and 9 for a second wash.
11. To the dry pellet, add 200 µl of wash buffer to each tube and vortex thoroughly.
12. Pipette 200 µl of 2% paraformaldehyde to each tube and vortex immediately. Immediate and thorough vortexing is vital to prevent fixing the cells in clumps.
13. Cells are now ready for acquisition and analysis. Fixed cells should be stable for up to 7 days stored capped at 2-8° C in the dark.

Indirect Staining Technique

1. Pipette sample into appropriately labeled tubes.
2. Centrifuge cells to pellet at 400 g for 5 minutes.
3. Aspirate and discard supernatant using vacuum apparatus. Avoid dislodging cell pellet.
4. Pipette appropriate amount of the primary (unconjugated) specific antibody or control antibody reagent. Volume will vary by manufacturer and titer.
5. Incubate 15 to 30 minutes. Incubation times longer than 15 minutes should be at 4° C for optimal staining.
6. Pipette 1 ml Wash Buffer to each tube, cap, and vortex.
7. Centrifuge tubes at 400 g for 5 minutes.
8. Aspirate and discard supernatant using vacuum apparatus. Avoid dislodging cell pellet.
9. Repeat steps 6, 7, and 8 for a second wash.
10. To dry pellet, pipette appropriate amount of secondary (conjugated) antibody to each tube.
11. Incubate 15 to 30 minutes at 4° C in the dark. Once fluorescent reagents have been added, protect the tubes from light to prevent fading.
12. Wash cells (steps 6, 7, and 8) two times.
13. To the dry pellet, add 200 µl of wash buffer to each tube and vortex thoroughly. Use 100 µl for 6 x 50 mm tubes.
14. Pipette 200 µl of 2% paraformaldehyde to each tube, cap and vortex immediately. Use 100 µl for 6 x 50 mm tubes. Immediate and thorough vortexing is vital to prevent fixing the cells in clumps.
15. Cells are now ready for acquisition and analysis. Fixed cells should be stable for up to 7 days stored capped at 2-8° C in the dark.

References

1. Bauer KD, Duque RE, and Shankey TV, eds: *Clinical Flow Cytometry: Principles and Applications*. Williams and Wilkins, p 634, 1993.
2. Bray RA, Landay AL, Identification and Functional Characterization of Mononuclear Cells by Flow Cytometry. Arch Path Lab Med 113:579, 1989.
3. Centers for Disease Control and Prevention: Guidelines for the Performance of CD4+ T-Cell Determinations in Persons with Human Immunodeficiency Virus Infection. MMWR 41:1, 1992.
4. Coligan JE, Kruisbeek AM, Marguiles DH, Shevack EM, Strober W, eds: The CD System of Leukocyte Surface Molecules. In: *Current Protocols in Immunology*, Vol. 2. Wiley and Sons: New York, p A.4.1, 1991.
5. Coon JS and Weinstein RS, eds: *Techniques in Diagnostic Pathology*, No. 2, Diagnostic Flow Cytometry; Williams and Wilkins, 1991.
6. Given AL: *Flow Cytometry: First Principles*. Wiley-Liss: New York, p 203, 1992.
7. Jackson AL, Warner NL: Preparation, staining, and analysis by flow cytometry of peripheral blood leukocytes. In: Manual of Clinical Immunology, 3rd ed., NR Rose and H Friedman, eds: American Society for Microbiology: Washington DC, p 226, 1986.
8. Landay AL, Ault KA, Bauer KD and Rabiniovitch PS eds: Clinical Flow Cytometry. Ann NY Acad Sci 677:468, 1993.
9. Riley RS, Mahin EJ, and Ross W: Clinical Applications of Flow Cytometry. Igaku-Shoin pub. New York-Tokyo. 1993.
10. Owens MA and Loken MR. Flow cytometry principles for clinical laboratory practice. Wiley-Liss: New York. 1995.
11. Leukocyte Typing V. Schlossman S. et al, eds. Oxford University Press, New York. 1995.

HLA-B27 Typing by Flow Cytometry

Anne M. Ward

Purpose

HLA-B27 is an antigen associated with the disease Ankylosing Spondylitis. Ninety percent (90%) of Caucasians with Ankylosing Spondylitis possess the B27 antigen. However, only twenty percent (20%) of people with the B27 antigen will develop the disease. Traditionally, B27 presence has been determined via the complement mediated microlymphocytotoxicity test using either locus specific trays or by complete serological typing. In comparison, the adaptation of the test to flow cytometry has provided a quick, easy to perform, and inexpensive means of detecting the B27 antigen. It is especially useful in testing large batches volumes of specimens.

Scope

The following procedure addresses sample preparation, sample analysis, data analysis, interpretation and troubleshooting. Comments concerning advantages and disadvantages are also included.

Introduction

The test consists of adding a monoclonal antibody to HLA-B27, conjugated with FITC fluorescent dye to whole blood or peripheral blood lymphocytes to form an antigen – antibody complex. After several washes to remove excess antibody, the sample is introduced into a flow cytometer, which measures light scatter and bound fluorescence of individual cells as they pass through a laser light source. The B27 antigen is defined as absent or present according to the percent of fluorescent-tagged lymphocytes, relative to positive and negative controls (mean or median channel shift).

Specimen

Five ml EDTA whole blood, Sodium Heparin, or ACD-A whole blood (<72 hours old)

Unacceptable Specimen

Specimens not maintained at room temperature
Clotted or hemolyzed specimens

Supplies and Equipment

Instrumentation

Flow Cytometer
Centrifuge
Vortex
2 µl pipette
100 µl pipette
12 x 75 mm test tubes (plastic or glass)

Reagents

One Lambda FITC Monoclonal Antibody (MoAb)
Whole blood lysing system, or
Ficoll-Hypaque density gradient medium for isolating peripheral blood lymphocytes
Fixing solution—Phosphate-buffered saline (PBS) with 0.5% formaldehyde

Quality Control

Reagent QC: A known HLA-B27 positive and a known HLA-B27 negative specimen must be run each day to ensure reagent stability.

Flow Cytometer QC: Prior to daily testing, the user must assure that the flow cytometer lase is aligned and that all running parameters are functioning within normal limits. Refer to the procedure in this manual for Flow Cytometer quality control (VI.C.1).

Procedure

A. Sample Preparation

1. Add 2 µl of B27 MoAb to test tube.
2. Add 100 µl of well-mixed whole blood or peripheral blood lymphocytes (1×10^6 cells/ml) to the appropriately labeled, corresponding tube.
3. Vortex and let sit between 10 min and 30 min at room temperature, away from bright light.
4. If whole blood is used, red cells must be lysed and pH balance restored. Resuspend in formaldehyde fixing solution.
5. The samples are ready to be analyzed using the flow cytometer or may be stored in the dark at 4°C for up to 24 hrs.
   Note: Results have shown increased differentiation between B27- and B27+ specimens, if incubated after whole blood lysis.

B. Sample Analysis

1. Align and quality control the flow cytometer according to the manufacturer's guidelines daily prior to testing samples.
2. Load the sample tube onto the cytometer and aspirate.
3. Gate to encompass the lymphocyte population. Count at least 2500 events.
4. Determine the fluorescence mean channel shift, relative to the negative control (MCS, see below).
5. Assign HLA-B27 phenotype as positive or negative based on the mean channel results (Figure 1).

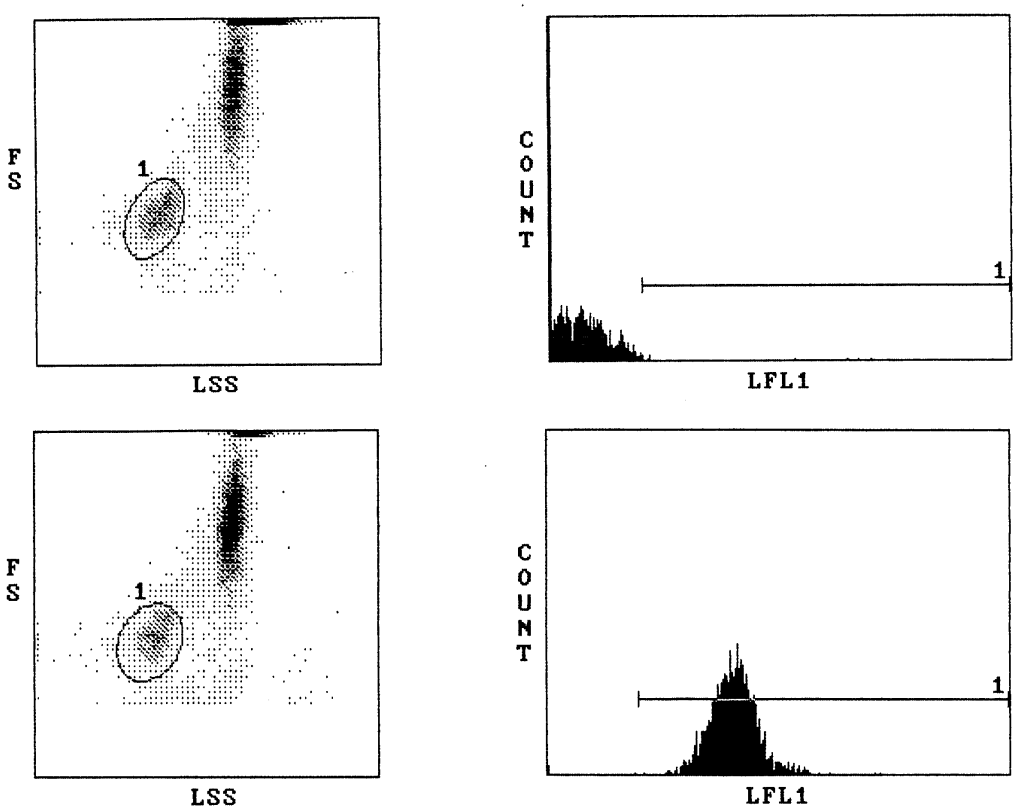


Figure 1: Examples of Flow Cytometry printouts.
Top: HLA-B27 Negative sample.
Bottom: HLA-B27 Positive sample; note the very obvious shift in fluorescence, relative to the negative patient.

C. Data Analysis

1. With each new lot number of B27 MoAb, 5 to 10 samples negative for the B27 antigen and 5 to 10 samples positive for the B27 antigen should be run to determine the appropriate mean channel range for positive and negative samples. Cutoffs vary due to the inherent variables encountered in the test, such as pipetting, whole blood lysis method, and type of flow cytometer, as well as alignment and standardization of instrument.

Example (Coulter/QPrep/...) mean channels:

B27$^-$	**B27$^+$**
0.30	3.57
0.29	6.91
0.57	5.42
0.27	3.76
0.31	8.34
Range = 0-0.60	Range ≥3.0

2. If the mean channel falls in between the ranges established, back-up testing such as microlymphocytotoxicity is recommended. Consistent results falling between the established ranges may indicate that a new mean channel range may have to be established.

Interpretation

A. Validation studies must be run to determine the percent of fluorescence that will be considered positive and negative for the HLA-B27 antigen in each laboratory. In our laboratory, 100 samples are run in parallel with the microlymphocytotoxicity test, in order to provide enough data to determine the cut off ranges.

B. Due to strong cross reactivity with other HLA antigens (such as HLA-B7) and depending on the source of B27 MoAb, there may be a "window" of false positive or false negative where accurate interpretation cannot be made using the flow cytometry method. Such samples must be repeated for a full Class I typing by molecular or serological methods.

C. Figure 2 demonstrates studies performed on over 300 samples using both Flow Cytometry with Genetic System MoAb and microlymphocytotoxicity testing.

The cutoff according to our studies is as follows:

HLA-B27 Negative is considered to be < 45% fluorescence.
HLA-B27 Positive is considered to be > 98% fluorescence.

D. Anything falling between 45% and 98% is set up by the microlymphocytotoxicity method. This "window" appears to be an expression of the cross reactivity with HLA-B7 approximately 75% of the time.

Note: One false negative sample by Flow Cytometry was observed out of 300 samples at 51.5% fluorescence. This sample was HLA-B27 Positive by microlymphocytotoxicity testing (Figure 2)

1. Different methods have been tested in order to decrease or eliminate the "window". None of these methods have made an improvement upon the number of samples which have to be set up by the microlymphocytotoxicity test. An HLA-B7/HLA-B27 dual staining monoclonal reagent may resolve this problem.
2. Over 3000 HLA-B27's have been tested by Flow Cytometry in our laboratory with approximately 300 samples requiring duplicate testing by the microlymphocytotoxicity method (approximately 10%). The One-Lambda MoAb has been found to greatly reduce false positive and false negative results.

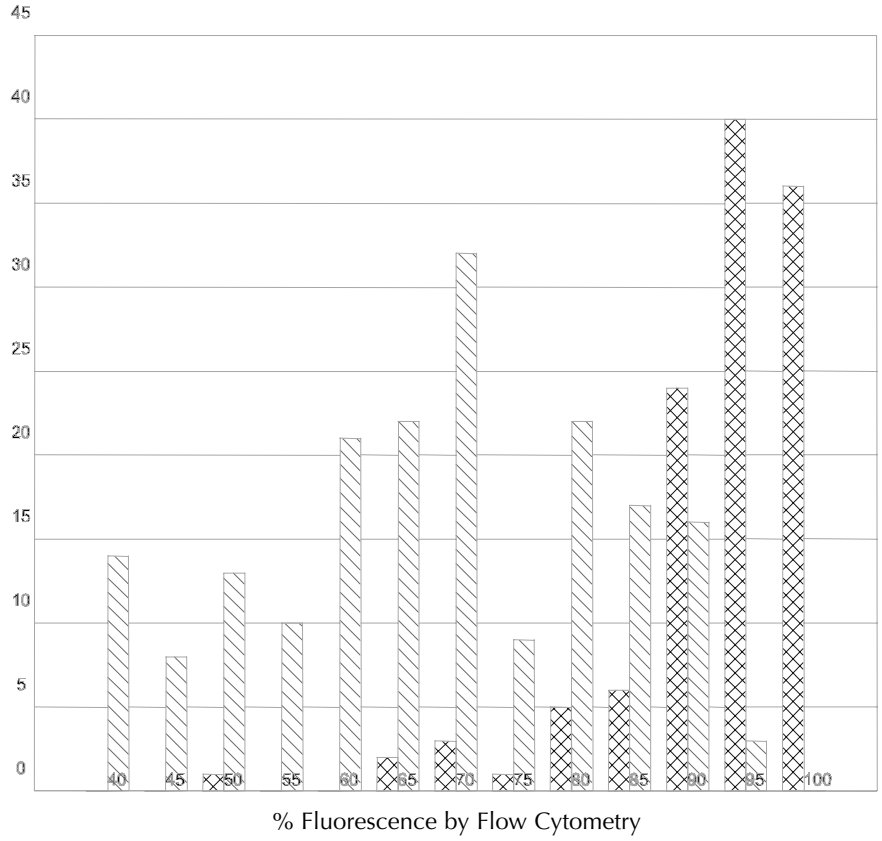


Figure 2. Comparison of HLA-B27 analysis by Flow Cytometry versus microlymphocytotoxicity testing. Double hatched bars represent HLA-B27 Positive samples and single hatched bars represent HLA-B27 Negative samples. The X axis is the percent fluorescence by flow cytometry and the Y axis, number of samples

Troubleshooting

A. If red cell contamination is evident after viewing the bitmap (gate), the test must be repeated, including repeating the lysing step and rerunning the sample. RBC contamination can be caused by an inadequate amount of lysing reagent administered to the tubes, or inability of reagent to lyse some patient's red cells (rare).
B. If the known negative or positive HLA-B27 quality control sample fails, another known sample should be run. If this sample also fails, reagent deterioration is likely. This is usually demonstrated by markedly decreased fluorescence in the HLA-B27 Positive Quality Control sample.

Note: The Q-prep must not be used for ficoll-hypaque isolated cells as it may result in false negative reactivity.

Advantages of HLA-B27 by Flow Cytometry

A. Reagent costs can be less than five dollars per patient, depending on volume.
B. Results are turned out quickly. Approximately ten samples can be turned out in less than an hour.

Disadvantages of HLA-B27 by Flow Cytometry

A. Purchasing a Flow Cytometer only for HLA-B27 testing may not be cost effective.
B. Retention of the HLA-B27 microlymphocytotoxicity method as a back-up.

References

1. *Coulter Cytometry Laboratory Manual*, Epics® Profile II Flow Cytometer: Coulter Corporation, Hialeah, FL (September, 1989).
2. Dei R, Arjomond-Shamsai M, Deng CT, Cesbron H, Bignon JD, Lee JH: A Monospecific HLA-B27 Fluoresceinisothiocyanate Conjugated Monoclonal Antibody for Flow Cytometry Typing. One Lambda, Inc., Canoga Park, CA, 1993.

CD34 Enumeration

M. Fran Keller and Lauralynn K. Lebeck

Purpose

Hematopoietic stem cell (HSC) transplantation is a clinical intervention used to reconstitute long-term multi-lineage hematopoiesis after intensive myeloablative therapy. Hematopoietic progenitor cells (HPC) as well as HSC are believed to express CD34. These CD34+ cells are a minor component of bone marrow (1-3%) and are also found in the peripheral blood of normal individuals, but at extremely low levels (0.04-0.1%). CD34+ cells can be mobilized from the marrow to the peripheral circulation in far greater numbers by chemotherapy and/or hematopoietic cytokines. This makes possible the use of peripheral blood progenitor cells (PBPC) versus bone marrow in both autologous and allogeneic transplant settings. The cellular composition of PBPC collections, however, are qualitatively different from bone marrow, with the former more likely to be influenced by factors such as methods of "mobilization," the clinical diagnosis, and the extent of exposure to prior therapy. The more recent use of human umbilical cord blood as a potential source of stem/progenitor cells adds yet another set of clinical variables.

The CD34+ population is heterogeneous, encompassing the earliest quiescent HSC as well as maturing, lineage-committed progenitors of all blood cell types. By using multi-parameter flow cytometry, it is possible to address not only quantitative aspects, but also qualitative composition of the stem/progenitor cell product. Additionally, since a flow cytometric analysis can be performed in less than 1 hour, it is suitable for the determination of optimal timing for apheresis collection and the "on-line" evaluation of the apheresis product.

Several flow cytometric methods have been described for CD34 enumeration. Additionally, commercial kits for CD34 staining and specific software programs are also available. The method described here is a basic two-color protocol that is recommended by ISHAGE (International Society of Hematotherapy and Graft Engineering) for CD34 enumeration.

Specimen

1. Cells (viability > 90%)
   a. Whole blood preserved in EDTA K_3 (purple top) maintained at room temperature. Specimen should be tested within 24 hours of the draw time.
   b. Bone marrow aspirate / apheresis product
   c. Frozen bone marrow / apheresis product may be used if sufficient pretest viability.

Reagents and Supplies

1. Wash buffer PBS with 0.1% NaN_3 and 5% Fetal Calf Serum (PBS/FCS)
   a. Add 0.1 g sodium azide for each 100 ml working PBS solution prepared to yield a final concentration of 0.1% sodium azide. (PBS Azide)
   SAFETY WARNING: Sodium Azide is toxic if inhaled. Wear a mask when handling undiluted material or use a chemical fume hood. Sodium Azide can be an explosion hazard in copper plumbing. Flush with large quantities of water when disposing of solutions in sink drains.
   b. Thaw and add one 5 ml aliquot of fetal calf serum for each 100 ml of working PBS Azide solution. See part a. above.
2. NH_4Cl lysing solution
   a. Stock 10x lysing reagent. Store at 4°C up to 1 month.
      1) To a 100 ml volumetric flask combine the following:
         1.0 g Potassium Bicarbonate (*Sigma Chemical Cat# P-9144*)
         8.26 g Ammonium Chloride (*Sigma Chemical Cat# A-5666*)
         0.037 g Ethylenediamine Tetraacetic Acid (EDTA) (*Sigma Chemical Cat# ED4S*)
      2) Dilute to 100 ml with distilled water and MIX well to dissolve components.
      3) Check pH. Should be 7.0 – 7.5
   b. Working 1x lysing reagent. Store at room temperature. Make **fresh** daily.
      1) Dilute the stock lysing reagent 1:10 with distilled water.
3. Fluorescence-conjugated antibodies
   a. CD45 FITC. The CD45 antigen is a family of glycoproteins with 5 isoforms. Each isoform can also be differentially glycosylated to produce a large number of glycoforms. Pan CD45 antibodies (hybridoma J33, 2D1, etc) that detect all isoforms and glycoforms are required to stain all nucleated white blood cells. By including only CD45+ events in the analysis, red blood cells, their nucleated precursors, platelets and cellular debris are excluded from subsequent analysis. Store at 4°C until expiration date.

   b. CD34 PE . The CD34 antigen is a family of differentially glycosylated structures. Class I epitopes are sensitive to both neuraminidase and glycoprotease. Class II epitopes are sensitive only to the glycoprotease, while class III epitopes are insensitive to both enzymes. Class I antibodies generate the most aberrant data in clinical samples, whereas class II and class III detect similar, but not identical numbers of CD34+ cells. It is important to use a CD34 antibody that detects all glycosylation variants of the molecule, i.e. class II or class III antibodies. QBEnd 10 hybridoma (class II, *Immunotech/Coulter*), 8G12 hybridoma (class III, *Becton Dickinson /PharMingen*), and 581 hybridoma (class III, *Immunotech /Coulter*) work interchangeably in the ISHAGE protocol. Store at 4ºC until expiration date.
   c. Isotype control PE. Based on the CD34 reagent used, an isotype control antibody should be stained as a negative control.
   Note: FITC and PE are light sensitive so keep in the dark.
4. 1% Formaldehyde solution.
   a. Stock solution is 10% formaldehyde (*Polysciences*). Make a 1:10 dilution of the stock in PBS Azide. pH 7.2 ± 0.2. Store in dark at 4°C. Stable for one month.
   SAFETY WARNING: Formaldehyde is a highly toxic carcinogen. Use personal protective equipment such as gloves, lab coat and mask when handling. Use of a fume hood is also recommended.
5. 12 x 75 mm Polystyrene Falcon tubes (*Fisher Cat# 352008*).
6. CD34 Control Cells. Several commercial reagents are available to quality control your staining protocol. (CD-Chex CD34, *Streck Laboratories*; Stem-Trol™, *Coulter;* CRISP CD34 Control Cells, *Phoenix Flow Systems*). Alternatively, the KG1a cell line can be used.

Instrumentation

1. Vacuum aspiration system
2. Channel Alarm Timer
3. Vortex Genie Mixer
4. Flow Cytometer
5. Refrigerated centrifuge
6. Test tube rack
7. Adjustable Pipettes: 10 µl to 100 µl
8. Repeating dispensers for delivering volumes from 500 µl to 2 ml

Calibration

Instruments such as repeating dispensers, centrifuges, timers, or temperature recording systems must be calibrated periodically to ensure that delivered volumes, centrifugation speed and time / temperature are consistent and accurate. Proper instrument setup and performance on the day of testing is critical for obtaining accurate and reliable results. The flow cytometer will have vendor specific standards such as beads that should be included each time the instrument is operated. Minimally, instrument settings such as photomultiplier tube (PMT) voltages, fluorescence compensation and sensitivity must be verified and recorded.

Quality Control

Due to the exquisite sensitivity afforded by this methodology, CD34 enumeration is particularly dependent on rigorous quality control measures for reagents and equipment including:

1. All specimen dilutions and volumes must be exact and accurate. Reverse pipetting technique, preferably with an automated pipettor is suggested.
2. The determination of the absolute CD34+ cell count in peripheral blood and apheresis products requires quantitation of the percentage of CD34+ cells in a specimen as determined by flow cytometry, as well as a nucleated cell count from an automated hematology analyzer (so-called two instrument platform analysis). Alternatively, by incorporating fluorescent beads in the flow cytometric analysis, an absolute CD34+ cell count can be generated with a single instrument platform. Single platform assays are highly recommended when absolute counts are desired. Standardized bead preparations such as *Becton Dickinson* TruCount Absolute Count Tubes or *Coulter* Stem-Count Fluorospheres require mandatory accuracy and precision when using these reagents.
3. It is critical in rare-event analysis to be able to discriminate the target from background noise or cellular debris. Progenitor cells stain dimly with CD45 therefore appropriate flow cytometry instrument set-up with high sensitivity is mandatory. Some protocols recommend a nucleic acid as the initial gating criteria (three-color protocol) to verify inclusion of all possible CD34+ cells.
4. Each new lot of CD45 and CD34 reagents must be tested prior to use to show that they stain the proper sub-population of cells. Testing in parallel with the current lot is the easiest method to evaluate new reagents.

Procedure

1. Ensure that the white blood cell (WBC) concentration is no greater than 30 x 10^9 WBC/L. Optimal concentration is 15 x 10^9 WBC/L. Dilute with PBS/Azide if necessary. Record the dilution factor for the calculation of the final CD34 absolute count.
2. Label 12 x 75 Falcon tubes for each sample including the control cells:
   a. Blank
   b. CD45 / Isotype control PE
   c. CD45 / CD34
   d. CD45 / CD34 duplicate
   Note: many laboratories do not perform testing in duplicate, however this is a very good indicator of technique and is strongly recommended .
3. Pipette 2 ml of sheath fluid (or PBS/Azide) into the BLANK tube. Set the tube aside.
4. Pipette 20 µl of each CD45-FITC and CD34-PE into tubes labeled as such. Add 20 µl each of CD45-FITC and Isotype control-PE to the appropriate tube.
5. Accurately pipette 100 µl of cell sample to the bottom of the three test tubes. Do not allow blood to remain on the inner tube walls. Remove traces with a cotton swab.
   Mix the cell preparation well before adding to ensure consistency from tube to tube.
6. Incubate for 20-30 minutes at room temperature. Protect from light.
7. Add 2 ml of 1x NH_4Cl lysing solution (except blank). Vortex immediately after each addition. Incubate at room temperature for 6 -10 minutes.
   a. Lyse/No wash technique. Tubes are ready to be acquired/analyzed by flow cytometry. This method is required for fluorobead single platform absolute count protocols. Samples must be analyzed within **1 hour**.
   b. Alternatively, a lyse/wash procedure can be utilized.
      i. Centrifuge 5 minutes at 500 x g, 4° C.
      ii. Aspirate supernatant. Resuspend pellet in 500 µl PBS/Azide.
      When aspirating do not aspirate cell pellet. Residual fluid volume should be < 30 µl.
      iii. While vortexing, add 500 µl of 1.0% formaldehyde to each tube.
      iv. Samples can be immediately analyzed or held at 4°C in the dark for up to 7 days.
8. Flow cytometric acquisition / analysis should be performed on a minimum of 75,000 CD45+ events / tube. These collection criteria should yield > 100 CD34+ cells. Pre-defined templates including cytometer settings are highly recommended for clinical use and can be easily defined for any of the commercial flow cytometers on the market. This greatly increases consistency between cytometer operators within your laboratory.
   a. Create Dot Plot 1 as FL1 CD45-FITC vs. Side Scatter. Create rectilinear region (R1) to include all CD45+ leukocytes and eliminate platelets, red blood cell debris, and aggregates. Display Gate 1 (G1 = R1) on Dot Plot 2.
   b. Create Dot Plot 2 as FL2 CD34-PE vs Side Scatter. Create rectilinear region (R2) on Dot Plot 2 to include all CD34+ events. Set a stop count of 75,000 events (CD45+ events) in Dot Plot 2. Display events from Regions 1 + 2 (Gate 2 = R2 and G1) on Dot Plot 3.
   c. Create Dot Plot 3 as FL1 CD45-FITC vs Side Scatter. Create amorphous region (R3) on Dot Plot 3 to include all clustered CD45+ dim events. Display events from Regions 1 + 2 + 3 (Gate 3 = R3 and G2) on Dot Plot 4.
   d. Create Dot Plot 4 as Forward Scatter vs. Side Scatter. Create amorphous Region 4 on Dot Plot 4 to include all clustered events with low SSC and intermediate to high FSC. Events from Region 1 + 2 + 3 + 4 (Gate 4 = R4 and G3) are real CD34+ HPC.

Results

An example of a lyse/wash two-color CD34 enumeration technique with corresponding dot plots and histograms is shown in Figure 1.

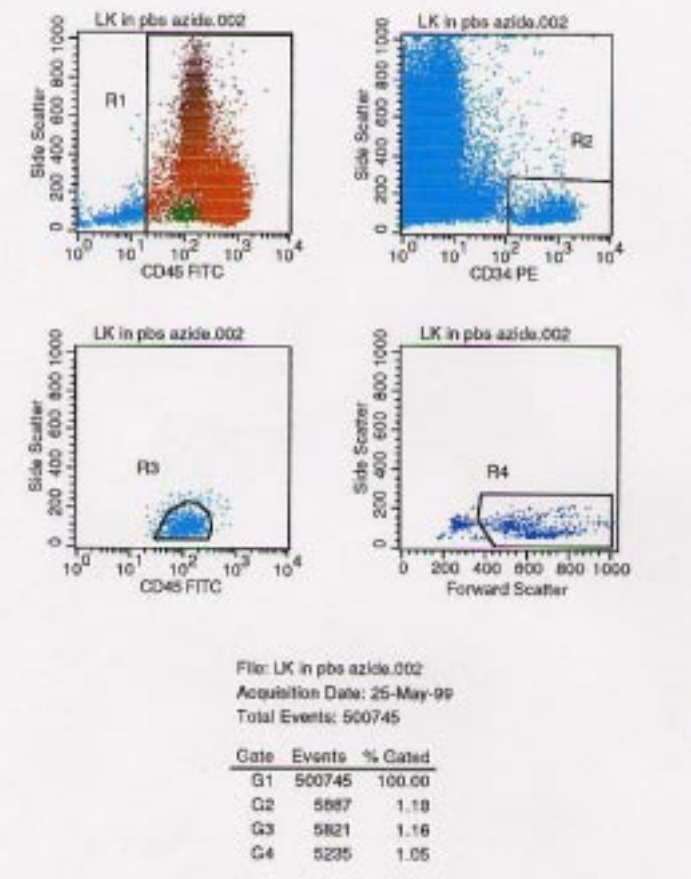


Figure 1. Enumeration of CD34+ cells in apheresis sample using CD45-FITC / CD34-PE. Plots 1-4 from Becton Dickinson FACSCalibur with Cellquest software. Plot 1 SSC versus FL-1 displays all events with a region (R1) defining CD45+ events. Plot 2 SSC versus FL-2 is gated on region R1. Plot 3 SSC versus FL-1 is gated on both R1 and R2 events and plot 4 SSC versus FSC includes only R1, R2 and R3 events.

Calculations

1. Average the results obtained from the duplicate specific CD45/CD34 tubes. The number of CD34+ HPC must fall within 10% of the mean for the duplicate samples.
2. Subtract the value obtained with the CD45/Control tube from the average CD34+ HPC value.
3. If the sample has been diluted, the result obtained above MUST be multiplied by the appropriate dilution factor. The final result obtained is the % CD34. If a single platform protocol has been used, the absolute count CD34 can be determined by the following formula:

$$\text{CD34+HPC Absolute Count (cells/µl)} = \frac{\text{Number CD34+ HPC counted}}{\text{Number of bead singlets counted}} \text{ X bead assay concentration}$$

4. For apheresis packs, the total number of CD34+ HPC per pack can be calculated by multiplying the HPC absolute value obtained above by the apheresis pack volume.

Procedure Notes

1. The "Milan" protocol is the earliest and most simple of the published procedures. It is a single color procedure, originally described by Siena et al. The gating strategy utilizes simple forward angle (FSC) versus side angle (SSC) light scatter to set a denominator. An isotype matched control is used in the traditional manner to set the positive analysis region for CD34+ cells. While some laboratories continue to define CD34 by this protocol, the two-color method that includes CD45 is highly recommended.
2. When evaluating alternate sources of HPC such as cord blood, CD45 is definitely needed as well as a single platform protocol for absolute count determinations. Many cord blood specimens have significant pre-B cell populations (CD10/CD19/CD34) that probably should be included in the CD34 enumeration but should be highlighted with a comment to the clinicians.

3. If the sample, regardless of source, is >24 hours old, a single platform protocol becomes mandatory for reproducible results.
4. Inclusion of viability dyes such as 7AAD are also highly recommended when testing frozen/thawed preparations.

Limitations of Procedure

If your laboratory procedure underestimates the number of CD34+ cells, this is okay for the patient. If however, you overestimate the CD34 value, it may hurt the patient. Consistent, precise enumeration is the most important if it is conservative.

References

1. Sutherland DR, Anderson L, Keeney M, et.al., The ISHAGE guidelines for CD34+ cell determination by flow cytometry. J. Hematotherapy 5:213, 1996.
2. Roth P, Maples J, Hall J, et.al., Use of control cells to standardize enumeration of CD34+ stem cells. Ann NY Acad. Sci. 770: 370, 1996.
3. Chin-Yee I, Keeney M, Anderson L, et.al. Current status of CD34+ cell analysis by flow cytometry: the ISHAGE guidelines. Clin. Immunol Newsletter 17:(2-3) 22, 1997.
4. Brecher ME, Sims L, Schmitz J, et.al., North American multicenter study on flow cytometric enumeration of CD34+ hematopoietic stem cells. J. Hematotherapy 5: 227, 1996.
5. Knape CC. Standardization of absolute CD34 cell enumeration. Letter to the Editor. J Hematotherapy 5:211, 1996.
6. Siena S, Bregni M, Belli N, et.al., Flow cytometry for clinical estimation of circulating hematopoietic progenitors for autologous transplantation in cancer patients. Blood 77:400, 1991.
7. Keeney M, Chin-Yee I, Weir K, et.al. Single platform flow cytometric absolute CD34+ cell counts based on the ISHAGE guidelines. Cytometry 34:61, 1998.
8. Nayar R, Keeney M, Weir K, et.al. Determining the absolute viable CD34+ cell count in post-cryopreservation cord blood samples using a single platform flow cytometry based on the ISHAGE guidelines (abstract). J Hematotherapy 7:280, 1998.

Flow Cytometric Detection of Intracellular Cytokine Production

Howard M. Gebel, John W. Ortegel, and Anat R. Tambur

Purpose

The chief obstacle to long-term allograft survival is immunological rejection. Unfortunately, the immune system of the recipient is unaware that the transplanted organ is beneficial and therefore responds in the fashion dictated by thousands of years of evolution, i.e., elimination of foreign (non-self) material. In simplest terms, an immune response is elicited when recipient T cells are activated by donor alloantigens. Antigen specific receptors on the surface of recipient T cells engage alloantigenic peptide fragments and transduce cytoplasmic signals which result in the production of cytokines. Cytokines are comprised of a large family of signaling proteins including interleukins (IL-1- 20), colony stimulating factors (e.g., GM-CSF), growth factors (e.g., VEGF), tumor necrosis factors (e.g., TNF-α), interferons (e.g., INF-γ), and chemokines (e.g., RANTES). Cytokines regulate cell function in autocrine, paracrine and/or endocrine fashion, binding with their specific cell surface receptors and initiating a cascade of intracellular signaling. Thus, post-transplant, the production of certain cytokines can promote clonal expansion and differentiation of alloantigen specific T lymphocytes which can then migrate to the site of the allograft. Experimental studies are beginning to explain how and to what degree various cytokines mediate clinical allograft responses ranging from tolerance to rejection. Such studies suggest that analysis of specific cytokine production by cells isolated from allograft recipients may be an approach that will identify patients at risk to develop acute and/or chronic rejection. Another application is the assessment of cytokine production at the single cell level to monitor the efficacy of immunosuppressive therapy in allograft recipients.

Specimen

Anticoagulated peripheral blood from allograft recipients and healthy controls. Specimens should be kept at room temperature and should arrive in the laboratory within 24 hrs of being drawn. Whole blood or isolated mononuclear cells can be analyzed.

Reagents and Supplies

1. Test tubes. Disposable 12 x 75mm polystyrene tubes or equivalent.
2. Staining Buffer
   a. Dulbecco's phosphate buffered saline (PBS)
   b. Heat-inactivated fetal bovine serum (FBS) to make a 5% solution
   c. Sodium azide to make a 0.09% (w/v) solution
   d. Adjust pH to 7.4 – 7.6, filter (0.2 mm pore membrane), and store at 4°C
3. Monoclonal antibodies. Any direct-conjugate fluorochrome is acceptable.
4. Polyclonal activators.
   Note: Cytokine production by normal resting cells is minimal. A supra-physiologic in-vitro stimulus is required in most circumstances to demonstrate the potential of cells to synthesize cytokines
   a. phorbol myristic acid (PMA; Sigma, Cat. # P-8139)
   b. calcium ionophore A23187 (Ionomycin, Sigma Cat. # C-9275)
   c. anti-CD28 (clone CD28.2, Pharmingen Cat # 33740D)
   d. anti-human CD3 (clone UCHT1, Pharmingen Cat. #030200D)
   e. phytohemagglutinin (PHA-P, Sigma Cat. # L9132), concanavalin A (Con-A, Sigma Cat. # C2010, or Staphylococcus enterotoxin B (Sigma Cat. # S-4881)
5. Intracellular inhibitors of protein transport.
   Note: Once synthesized, cytokines are rapidly exported from the Golgi apparatus. Inhibiting their transport promotes intracellular accumulation and thereby facilitates detection. Be aware that transport inhibitors may decrease the expression of surface antigens used to identify cell subsets
   a. brefeldin A (GolgiPlug™ Pharmingen, Cat. # 2301KZ)
   b. monensin (GolgiStop™ Pharmingen, Cat. #2092KZ)

6. Permeabilization solution.
   a. saponin (purified, Sigma Cat. # S-4521)
   b. Hanks Balanced Salt Solution (HBSS) with 0.01 M HEPES buffer
      1) Prepare a 0.05 – 0.1% saponin solution in HBSS with HEPES
      2) Store at 4°C.

   Note: Saponins are glycosides made up from a steroid body attached to a hydrophilic carbohydrate chain. Saponins intercalate into the cell membrane via their high affinity for and contact with chololesterols, forming ring-shape complexes with a central pore approximately 8nm in diameter. Pore formation is reversible, meaning that saponin must be continuously present until the procedure is completed.
7. Fixative **Caution: Carcinogenic**
   a. paraformaldehyde (PFA; Sigma)
   b. Sodium Phosphate Monobasic (NaH_2PO_4)
   c. Sodium Hydroxide (NaOH)
   d. glucose
   e. distilled water
      1) Prepare Phosphate buffer: Sodium Hydroxide (NaOH) 3.85 g/L ; (NaH_2PO_4) 16.833 g/L; glucose 5.4 g/L; QS with water.
      2) Make a 4% solution of paraformaldehyde in phosphate buffer. Heat the mixture with constant stirring under a chemical hood.
      3) Adjust pH to 7.4 store at 4°C.
8. HBSS made to 0.1% BSA (Sigma Cat. # 2153)
9. Tissue culture plates (optional)
10. Cytokine antibodies and recombinant cytokines.

    Note: These cytokines were chosen solely as examples and are not intended to be a complete list. The following are Pharmingen products; designations refer to their catalog numbers.

Recombinant Specificity	Clone	FITC	PE	APC	Unlabeled	Cytokine
IL-2	MQ1-17H12	18954A	18955A	18959A	18951A	9621T
IL-4	8D4-8		18655A		18651A	19641V
IL-10	JES3-19F1		20705A	20709A	20701A	19701V
IFN-γ	827	20664A	20665A	20669A	20661A	19751G
TNF-α	MAb11	18644A	18645A	18649A	18641A	19761T

Instrumentation

1. Vacuum aspirator
2. Channel Alarm Timer
3. Vortex Genie Mixer
4. 37°C incubator with 5% CO2
5. Refrigerated centrifuge
6. Flow Cytometer
7. Hemacytometer – Coverslips
8. Adjustable Pipettes: 1-20 µl and 10-200 µl
9. Repeating dispensers for delivering volumes from 100 µl to 5 ml
10. Microscope

Calibration

Proper instrument setup is critical for obtaining accurate and reliable results. To calibrate the flow cytometer refer to the procedure manual provided by the manufacturer. Minimally, instrument settings such as photomultiplier tube (PMT) voltages, fluorescence compensation and sensitivity must be verified and recorded on a daily basis. Data is acquired using appropriate software and displayed as dot plots (two color) or histograms (single color). Cytokine expression is generally reported as the percentage of cells staining for intracellular cytokine(s).

Quality Control

To assure the conditions are appropriate, positive and negative controls for each cytokine evaluated must be incorporated into the assay.

1. A commercial source (e.g., Pharmingen, see below) of activated and fixed cells can be utilized to document the reliability of the fluorochrome conjugated anti-cytokine reagents.

Cell Set	Cat.#	Cytokines Measured
HiCK-1	23261Z	IL-2, IFN-γ, TNF-α
HiCK-2	23262Z	IL-3, IL-4, IL-10, IL-13, GM-CSF

2. Frozen cells from donors previously shown to produce cytokine upon activation can be utilized as additional controls to document activation conditions, although the routine incorporation of fresh cells from a walking panel of normal healthy controls will suffice.
3. There are three different types of negative controls appropriate.
   a. Stain cells with a fluorochrome conjugated irrelevant isotype control antibody
   b. Pre-incubate fluorochrome conjugated anti-cytokine antibody with recombinant cytokine (blocking). Intracellular cytokine staining techniques and the use of blocking controls are described in detail by C. Prussin and D. Metcalfe.[9]
   c. Pre-incubate cells with unconjugated antibody before staining with the fluorochrome conjugated anti-cytokine antibody. These controls will allow the investigator to distinguish between specific or non-specific intracellular staining.

Procedure

1. Isolation of mononuclear cells from anticoagulated peripheral blood should follow routine protocols of the laboratory. Adjust cell concentration to 1.0 X 10^6 cells/ml in RPMI supplemented with 5% FBS.
2. Pipette 1 ml of 1.0 x 10^6 cells/ml into the appropriate tissue culture plate wells.
3. Add 20 ng/ml PMA, 1mM ionomycin and 3mM monensin to each well and incubate for 6 hours at 37°C in 5% CO_2.
4. Cells should be washed several times in HBSS before fixation to remove any residual protein from the culture medium. For each wash: centrifuge at 400g; 2-8°C for 5 minutes. Aspirate the supernatant, add fresh HBSS, and vortex. Repeat several times.
5. Adjust cell concentration to 1.0 X 10^6 cells/ml. Aliquot 500 ml / tube. Number of tubes will be determined by the number of cell populations to be analyzed. Centrifuge tubes to generate a cell button and gently vortex.
6. Add an optimal concentration (usually 20 µl) of the required lineage monoclonal antibody, specific for the cell surface antigen, such as CD4, CD8, CD20, CD16 etc. Use an antibody directly conjugated with fluorochrome. Incubate at 4°C for 30 minutes.
7. Wash cells twice with HBSS, as in step 4, and proceed to the fixation / permeabilization steps.
8. While vortexing the tube, add 0.5 ml of 4% PFA, and incubate for 5 minutes at room temperature with occasional agitation to avoid cell aggregation.
9. To the fixed cell suspension, add 4 ml of ice-cold HBSS supplemented with 0.1% BSA.
10. Wash cells with HBSS-saponin to enable permeabilization. Thereafter, all staining and washing procedures must be performed in the presence of saponin.
11. Distribute the fixed cells into three tubes. Add a predetermined optimal concentration [commercial antibodies – follow manufacturer's instruction; otherwise, these have to be experimentally determined. Volume of reagent should be minimal] of the fluorochrome conjugated anti-cytokine antibody to tube 1 and irrelevant isotype control antibody to tube 2. The cells in tube 3 should be incubated with unconjugated anti-cytokine antibody in a concentration identical to tube 1. Incubate at 4°C for 30 minutes in the dark.
12. Wash cells twice using HBSS-saponin solution.
13. Resuspend cells in tubes 1 and 2 in HBSS supplemented with 0.1% BSA. These cells are ready for flow cytometry analysis.
14. To the cells in tube 3, the fluorochrome conjugated anti-cytokine antibody should be added at the appropriate concentration. Incubate at 4°C for 30 minutes in the dark.
15. Wash cells twice using HBSS-saponin solution and resuspend in HBSS supplemented with 0.1% BSA. Analyze these cells by flow cytometry to confirm that the fluorochrome conjugated cytokine antibody has been blocked from binding to the intracellular cytokine.
16. Positive and negative controls should be analyzed first to evaluate the validity of the test. Quadrant and histograms markers should be set based on the negative controls.

Calculations

Lymphocytes are analyzed by placing logical gates or regions around the cell population of interest. Using the appropriate negative control(s), fluorochrome cursors are situated such that no cells appear in the positive region or quadrant. The percentage of positive cells (upper right quadrant-double positive cells; cells that express the surface antigen of inter-

est plus the intracellular cytokine being examined) are then determined using statistical analysis software supplied by the flow cytometer manufacturer.

Results

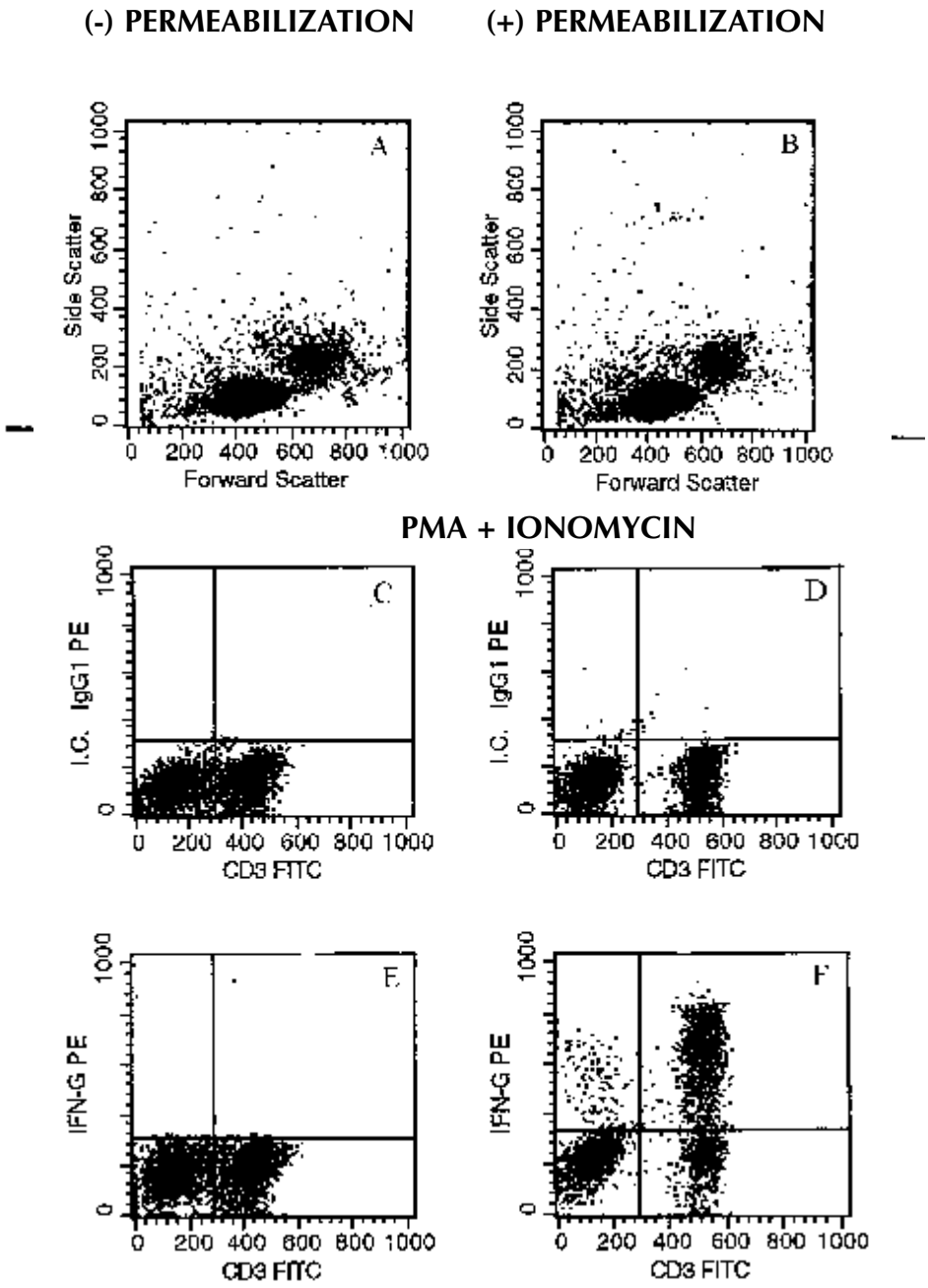


Figure 1. The effect of permeabilization solution on cellular light scatter properties, cell surface antigen staining, and intracellular cytokine staining. *A and B* are the forward and side light scatter profiles of normal human peripheral blood mononuclear cells cultured in media for 6 hours. Cells in *A* were not permeabilized, whereas cells in B were permeabilized with buffered saline containing saponin (0.1%) prior to antibody staining. *Note: While no difference is detected between permeabilized and non-permeabilized samples of non-activated cells, forward and/or side light scatter properties of cells incubated with different biological activators may be altered. C-F* represent cells from the lymphocyte gated populations of A or B following their activation with PMA (50 ng/ml) and ionomycin (1μM) for 6 hours. C and E are activated cells that were not permeabilized; D and F are activated cells which were permeabilized prior to antibody staining. C and D are negative controls (PE-conjugated irrelevant antibody isotype matched to the PE-conjugated anti-cytokine antibody) to assess background staining. E and F were stained with PE-conjugated anti-IFN-γ. *Note: While the permeabilization solution did not alter the expression of CD3 on the surface of these cells, some biological activators may down regulate the expression of certain surface antigens.* Cells in the upper right quadrant represent those cells positive for CD3 and intracellular IFN-γ.

EXPRESSION OF TNF-ALPHA BY CD3+ CELLS FROM THE PERIPHERAL BLOOD

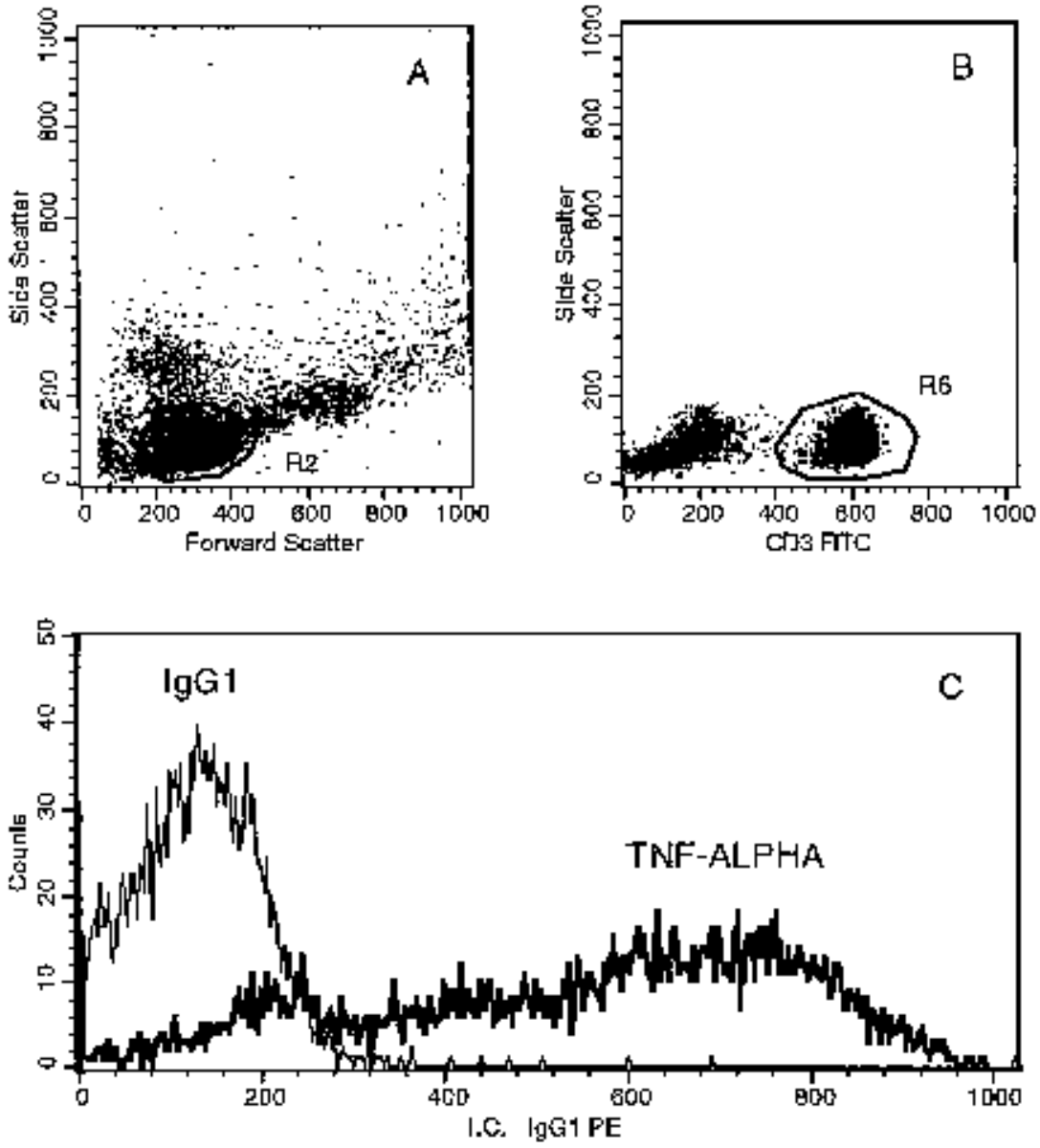


Figure 2. Expression of TNF-α by CD3+ PBMC.
Normal human PBMC were activated with PMA (50 ng/ml) and ionomycin (1mM) for 6 hours prior to antibody staining. Cells were fixed, permeabilized and stained as described. A represents the forward and side light scatter profile of the activated lymphocytes: *B* displays CD3 positive cells which are then gated and used for subsequent analyses; *C* represents TNF-α expression of the CD3 gated cells from B as compared with background (isotype matched irrelevant FITC-conjugated antibody).

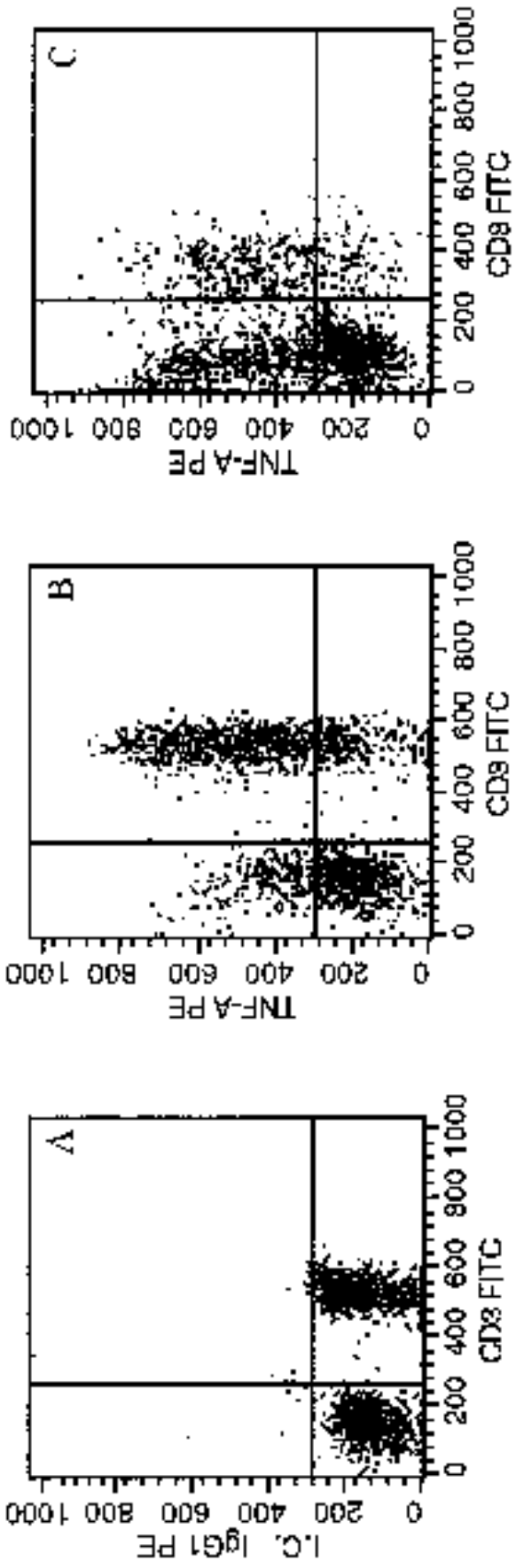


Figure 3. CD3+ and CD8+ lymphocytes display intracellular TNF-α after activation with PMA and ionomycin. Normal human PBMC were activated with PMA (50 ng/ml) and ionomycin (1mM) for 6 hours prior to antibody staining. Cells were fixed, permeabilized and stained as described. *A-C* represent gated lymphocytes stained with antibodies specific for either total T cells (CD3) or the CD8 subset of T cells. A represents cells stained with anti-CD3 and the appropriate isotype matched irrelevant control antibody (no cells in the upper right quadrant); B represents cells stained with anti-CD3 and anti-TNF-α (double positive cells in the upper right quadrant); C represents cells stained with anti-CD8 and anti-TNF-α (double positive cells in the upper right quadrant).

Procedure Notes

Numerous variations on staining protocols are available. Each laboratory should evaluate and determine the most appropriate approach to be used for their particular applications.

Limitations of Procedure

A major limitation of the current assays being used to detect intracellular cytokines is that unstimulated cells (at least from normal peripheral blood) do not have detectable levels of intracellular cytokines. The only way to detect these cytokines is via in vitro activation. This adds an artificial component to the assay that could easily explain patient to patient variation. Furthermore, this assay will only determine what percentage of a given cell population produces the cytokine(s) under study. The procedure is unable to quantify how much cytokine(s) is being produced per cell. Since polymorphisms in cytokine genes (e.g., those encoding for TNF-α, INF-γ and IL-10) differentiate individuals as high or low producers, it is certainly conceivable that a high producer with a small percentage of cells producing TNF-α may have a much higher risk of rejection than a low producing individual with twice as many TNF-α producing cells.

Another factor to consider when applying this assay to immune status evaluation of allograft recipients is that in initial studies in humans, rejection episodes (acute and chronic) or immunological quiescence are not apparently restricted to the cytokine patterns defined in experimental models (i.e., the type 1/type 2 T helper cell paradigm). This lack of association may be caused exclusively by the immunosuppressive regimen, but more likely, is the consequence of the almost limitless diversity among donor/recipient pairs. For example, polymorphisms in just cytokine genes mentioned above may play a central role in how a given patient responds immunologically to an allograft.

References

1. Assenmacher, M., J. Schmitz and A. Radbruch. 1994. Flow cytometric determination of cytokines in activated murine T helper lymphocytes: expression of interleukin-10 in interferon-γ and in interleukin-4-expressing cells. *Eur j. Immunol.* 24:1097-1101.
2. Carter, L. L., and S.L. Swain. 1997. Single cell analyses of cytokine production . *Immunol.* 9:1 77-182.
3. Ferrick, D. A., M. D. Schrenzel, T. Mulvania, B. Hsieh, W. G. Ferlin and H. Lepper. 1995. Differential production of interferon-γ and interleukin-4 in response to Thl – and Th2-stimulating pathogens by gd T cells in vivo. *Nature.* 373:255-257.
4. Jung, T., U. Schauer, C. Heusser, C. Neumann and C. Rieger. 1993. Detection of intracellular cytokines by flow cytometry. *J. Immunol Meth.* 159:197-207.
5. Nickerson, P., W. Steurer, J. Steiger, X. Zheng, A. W. Steele, and T. B. Strom. 1994. Cytokines and the Th1/Th2 paradigm in transplantation. *Curr. Opin. Immunol.* 6:757764.
6. O'Mahony, L., J. Holland, J. Jackson, C. Feighery, T. Hennessy and K. Mealy.1998.Quantitative intracellular cytokine measurement: age-related changes in proinflammatory cytokine production. *Clin. Exp. Immunol.*113:213-219.
7. Parks, D. R., L. A. Herzenberg, and L. A. Herzenberg. 1989. Flow cytometry and fluorescence activated cell sorting. In *Fundamental Immunology*, 2nd Edition. W. E. Paul, ed. Raven Press Ltd., New York, p. 781-802.
8. Picker, L. J., M. K. Singh, Z. Zdraveski, J. R. Treer, S. L. Waldrop, P R. Bergstresser, and V. C. Maino. 1995. Direct demonstration of cytokine synthesis heterogeneity among human memory/effector T cells by flow cytometry. *Blood.* 86:1408-1419.
9. Prussin, C. and D. Metcalfe. 1995. Detection of intracytoplasmic cytokine using flow cytometry and directly conjugated anti-cytokine antibodies. *J. Immunol Meth.* 188: 117-128.
10. Rosenberg, A. S. and A. Singer. 1992. Cellular basis of skin allograft rejection: an in vivo model of immunemediated tissue destruction. [Review]. *Annu. Rev. Immunol.* 10:333358.
11. Sander, B., J. Andersson and U. Andersson. 1991. Assessment of cytokines by immunofluorescence and the paraformaldehyde-saponin procedure. *Immunol. Rev.* 119:65-93.
12. Sewell, W. A., M. E. North, A. D. Webster and J. Farrant. 1997. Determination of intracellular cytokines by flow cytometry following whole blood culture. *J. Immunol. Meth.* 209:67-74.
13. Tkaczuk, J., L. Rostaing, O. Puyoo, C. Peres, M. Abbal, D. Durand, and E. Ohayon.1998.Flow Cytometry of intracytoplasmic cytokines after neoral or sirolimus intake is an informative tool for monitoring in vivo immunosuppressive efficacy in renal transplant recipients. *Transplantation Proc.* 30:2400-2401.
14. Van Den Berg, A. P., W. N. Twilhaar, G. Mesander, W. J. van Son, W. van der Bij, I. J.Klompmaker, J. H. Slooff, T. H. The, and L. H. de Leij. 1998. Quantitation of immunosuppression by flow cytometric measurement of the capacity of T cells for interleukin-2 production. *Transplantation* 65(8):1066-1071.
15. Vikingson, A., K. Pederson and D. Muller. 1994. Enumeration of IFN-γ producing lymphocytes by flow cytometry and correlation with quantitative measurement of IFN-γ. *J. Immunol Meth.* 1 73:219-228.
16. Weiss, A. and D. R. Littman. 1994. Signal transduction by lymphocyte antigen receptors. *Cell* 76:263274.

Quantitative Plasma OKT3 Levels

Leah N. Hartung and Carl T. Wittwer

Purpose

Murine monoclonal antibody OKT3 is used for the prophylaxis and treatment of transplant rejection. OKT3 is specific for CD3, the T-cell antigen receptor. Administration of the drug results in the depletion of T lymphocytes from the peripheral blood within minutes. When transplant patients are injected with OKT3, a residual amount of unbound OKT3 remains circulating. The unbound product can be quantified by flow cytometry. The method described is an indirect immunofluorescence assay utilizing human mononuclear cells as a carrier of CD3 to bind free plasma OKT3. The cells are incubated with the patient's plasma and then labeled with fluorescein-conjugated goat anti-mouse immunoglobulin antibody. By comparing the mean fluorescence of patient samples to that of OKT3 standards, the amount of circulating OKT3 can be quantified.

Specimen

Samples are usually drawn prior to OKT3 injection. Plasma from a heparinized tube (green top) is optimal and serum from a clot tube (red top) is acceptable. Samples should be submitted at room temperature unless transport is necessary. If shipping is required, centrifuge and remove 12 ml of plasma or serum and keep refrigerated. Contaminated samples or samples greater than 48 hours old are unacceptable.

Reagents and Supplies

Nalgene sterilization filter units – 500 ml 0.45 µm filter membrane
Microcentrifuge tubes with snap cap – 1.5 ml
Nalgene cryogenic boxes – 10 x 10
Nalgene cryogenic controlled-rate freezing containers – 18 place
Cryogenic vials – 2.0 ml
Glass Beakers 600 ml up to 2000 ml capacity
Carboy with spigot – 25 L
Magnetic stir bar
Test tubes
a. Polystyrene 12 x 75 mm
b. Borosilicate 16 x 125 mm
c. Borosilicate 13 x 100 mm
Conical tubes 50 ml
Orthoclone OKT3 (Ortho Pharmaceutical) 1 mg/ml
RPMI 1640 (buffered) with L-glutamine and without sodium bicarbonate
Fetal bovine serum (FBS)
HEPES – free acid 99.5% (titration) (N-[Hydroxyethyl]piperazine-N'-[4-butanesulfonic acid]) C_8,H_{18},N_20_4S; FW 238.3
Sodium bicarbonate solution (7.5%) $NaHCO_3$
Sodium phosphate dibasic anhydrous (Na_2HPO_4); FW 142.0 anhydrous crystalline
Potassium phosphate monobasic (KH_2PO_4); FW 136.1 anhydrous crystalline
Sodium chloride (NaCI); FW 58.44 >99.5%
Sodium hydroxide (NaOH); FW 40.00 anhydrous pellets minimum 98%
Hydrochloric acid (HCI); FW 36.46 1 N
Goat anti-mouse immunoglobulin-FITC (GAM-FITC) – Total IgG and IgM specificity (i.e., Beckman Coulter Cat# 6602159)
Paraformaldehyde, crystalline
Histopaque™-1077
Dimethyl sulfoxide (DMSO) (C_2H_6SO); FW 78.13 minimum 99.5%
Ethylenediaminetetraacetic acid (EDTA) trisodium salt: hydrate ($C_{10}H_{12}N_20_8Na_4$); FW 380.2
Bovine albumin fraction V powder – minimum 96%
Whole blood lysing reagent kit – (i.e., Beckman Coulter Cat# 6602764)

Preparation of Reagents

1. RPMl 1640 (buffered) to be used in Diluting media, Wash media, and Freezing dia. Stable for 30 days when stored at 4°C.
   a. QS a vial of RPMI 1640 to 1 liter with distilled water in 2 liter beaker.
   b. Add 4.5 ml sodium bicarbonate ($NaHCO_3$)
   c. Add 4.76 g HEPES.
   d. Adjust pH to 7.2 (± 0.1) with 1N HCI or 1N NaOH.
   e. Sterile filter the medium into two 500 ml Nalgene filter flasks.
2. RPMI 1640 Diluting Media with 2% FBS. Stable for 30 days when stored at 4°C.
   a. Add 490 ml RPMI 1640 (buffered) to 600 ml beaker.
   b. Add 10 ml fetal bovine serum to RPMI 1640.
   c. Sterile filter into 500 ml Nalgene filter unit.
3. RPMI 1640 Wash Media with 10% FBS. Stable for 30 days when stored at 4°C.
   a. Add 450 ml RPMI 1640 (buffered) to 600 ml beaker.
   b. Add 50 ml fetal bovine serum.
   c. Sterile filter into 500 ml Nalgene filter unit.
4. RPMI 1640 Freezing Media. Stable for 30 days when stored at 4°C.
   a. Add 400 ml RPMI 1640 (buffered) to 600 ml beaker.
   b. Add 50 ml of fetal bovine serum.
   c. Add 50 ml of DMSO (dimethyl sulfoxide).
   d. Sterile filter into 500 ml Nalgene filter unit.
5. Goat anti-mouse FITC (GAM-FITC). Refer to label for expiration.
   a. Reconstitute lyophilized reagent with 500 µl distilled water.
   b. Dilute the reconstituted antisera in RPMI 1640 Diluting Media. A titration (approximately 1:60) should be done with each new lot to determine optimum fluorescence intensity.
   c. Batches may be diluted, aliquoted into 1.5 ml microcentrifuge tubes and stored at –20°C until used.
6. Phosphate Buffered Saline (PBS) – Store at room temperature for 30 days; recheck pH at this time.
   a. Place 2 liters of deionized water in beaker with a magnetic stir bar.
   b. Slowly dissolve 45.4 g sodium phosphate dibasic anhydrous (Na_2HPO_4),16.4 g potassium phosphate monobasic (KH_2PO_4), and 140 g sodium chloride (NaCI) in the water.
   c. Add the 2 liter solution to a 25 liter carboy. Add 18 liters of deionized water. Adjust the pH of the final solution to 7.3 (± 0. 1) with 1N HCI or 1N NaOH.
7. 2% Paraformaldehyde. Store at 4°C. Stable for 30 days.
   Caution: This reagent must be prepared inside a fume hood.
   a. Heat 800 ml of sterile PBS to 60°C.
   b. Add 20 g of paraformaldehyde. Mix with a stir bar.
   c. Add 10 N NaOH one drop at a time until solution clears.
   d. Cool the solution to room temperature.
   e. Adjust pH to 7.4 with IN HCI.
   f. Q.S. to 1 liter with sterile PBS.
8. OKT3 Standards. Aliquot 500 µl of standards into appropriately labeled microcentrifuge tubes and store at -70°C. Working standards can be stored at 4°C for 7 days.
   a. Make stock solution of OKT3 (1000 ng/ml) by making a 1: 1000 dilution of the 1 mg/ml solution in FBS (place 5 µl of OKT3 [1 mg/ml solution] in a 13 x 100 mm test tube add 4.995 ml of FBS).
   b. For 50 ng/ml, 300 ng/ml and 600 ng/ml standards prepare as follows:
      50 ng/ml (add 50 µl stock + 950 µl FBS)
      300 ng/ml (add 300 µl stock + 700 µl FBS)
      600 ng/ml (600 µl stock + 400 µl FBS)
   c. For the 0 ng/ml standard use FBS,
9. Phosphate buffered saline with EDTA (PEB). Store at 4°C for 30 days.
   a. Place 1 liter of PBS in a 2 liter beaker with magnetic stir bar.
   b. Add 1.92 g of EDTA.
   c. Add 2.5 g of bovine serum albumin.
   e. Mix until all reagents have gone into solution.
10. Lysing solution
    a. To a 16 x 125mm test tube at 6 ml of PBS.
    b. Add 250 µl Beckman Coulter lysing solution.
    c. Cap and mix thoroughly.
11. Cryopreserved mononuclear cell preparation. Store at -70°C for 6 months.
    a. Transfer the buffy coat from a whole blood unit into sterile sodium heparin tubes (green top).
    b. Rock tubes gently for 5 minutes to thoroughly mix.
    c. Dilute blood 1:10 with PEB in 50 ml conical tubes.
    d. Centrifuge at 200 x g for 12 minutes.

e. Aspirate the supernatant leaving 2 – 5 ml on the cell pellet. Repeat steps b and c.
f. Dilute washed cells 1:2 with PEB.
g. Layer 10 ml of diluted blood over 4 ml of Histopaque™ in a 16 x 125 mm tube.
h. Centrifuge at 400 x g for 40 minutes.
i. Remove the mononuclear layer to 50 ml conical tubes. Dilute 1:4 with PEB.
j. Centrifuge at 400 x g for 10 minutes. Aspirate supernatant.
k. Resuspend each pellet in 2 ml PEB. Transfer to 1 conical tube.
l. Add 2 -3 ml lysing solution. Gently vortex.

Note: DO NOT allow lysing solution to stand on the cells for more than 30 seconds.

m. Immediately wash with 20 – 40 ml of PEB.
n. Centrifuge at 400 x g for 10 minutes. Aspirate supernatant.
o. Repeat steps m and n.
p. Resuspend in RPMI Freezing – Medium. (Approximately 10 ml).
q. Quickly count cell concentration on a hemacytometer and adjust to 1 x 10^7 cells/ml with RPMI freezing media.
r. Quickly pipette 1 ml aliquots into sterile cryovials. Place in cryogenic controlled-rate freezing containers and freeze to -20°C for 24 hours then transfer frozen vials to cryogenic boxes and store at -70°C.

Instrumentation/Equipment

Electronic or Top Loading Balance (Suggestion: electronic balance, CMS # 01-914-112)
pH Meter
Hotplate
Flow Cytometer

Note: Precaution must be taken to avoid exposure to laser radiation.

Calibration

OKT3 Standards – Known concentration standards are used to plot a standard curve of mean fluorescence vs. OKT3 concentration. Patient plasma values are interpolated from this standard curve.

Quality Control

1. The negative control for this assay is fetal bovine serum or any plasma from an untreated individual.
2. The positive control for this assay is pooled, OKT3-treated patient plasma that has been assayed and a mean value calculated. A positive control should be analyzed with each run and have an OKT3 concentration of 300 ng/ml ± 100 ng/ml.
3. Fetal bovine serum or an untreated individual can be used as the "0" standard (i.e., 0 ng/ml). The mean channel of the 0 standard should not exceed the 50 ng/ml standard or any patient sample evaluated.
4. If the correlation coefficient of the standard curve is lower than 0.950 the assay should be rejected and repeated.

Procedure

Note: This procedure has been developed with an EPICS XL/ML – Beckman Coulter and can be used as a point of departure for other Coulter models or other vendors. However, the test must be validated for these other cytometers, prior to actual patient testing.

1. Plasma/serum separation
   a. Patient samples are centrifuged at 400 x g for 5 minutes and plasma separated.
   Note: It may be necessary to spin the separated plasma again at 600 x g for 3 minutes to eliminate RBC contamination.

2. Mononuclear cell harvesting
   a. Quickly thaw an aliquot of mononuclear cells by immersing the vial in a 37°C water bath for approximately 1 minute or until completely thawed.
   b. Transfer cells to a 16 x 125 mm test tube and resuspended in 10 ml of RPMI *Cell Wash Medium.*
   c. Centrifuge at 300 x g for 6 minutes.
   d. Decant the supernatant and resuspend cell pellet in 1 ml *Wash Media*.
   e. Determine the cell concentration on a hemacytometer and adjust to 1.0 x 10^7 cells/ml with *Wash Media.*

3. OKT3 quantitation
   a. To labeled 12 x 75 mm test tubes, add 5 µl of patient serum, OKT3 standard, or control. (If patient's OKT3 level is expected to be > 600 ng/ml, make an appropriate dilution with fetal calf serum in a separate 12 x 75 test tube. Use 5 µl of this dilution.)
   b. Pipet 100 µl washed mononuclear cells into each tube and vortex gently.
   c. Incubate for 15 minutes at room temperature.

d. Wash with 3 ml of PBS and centrifuge at 600 x g for 3 minutes.
e. Aspirate the supernatant completely down to the cell pellet, being careful not to disturb or aspirate pellet.
f. Add 100 µl working GAM-FITC to each tube.
g. Incubate for 15 minutes at 4°C in the dark.
h. Add 3 ml of PBS to each tube and centrifuge at 600 x g for 3 minutes. Repeat.
i. Aspirate to cell pellet on final wash step.
j. Add 500 µl of 2% paraformaldehyde and vortex gently.
k. Keep samples refrigerated until analysis.
l. Analyze samples on the flow cytometer, collecting at least 3,000 events (lymphocytes by light scatter) and acquiring log green fluorescence (FL1). Adjust the PMTs and/or gains so that all standard peaks are on scale. A four decade log scale provides optimal resolution of the OKT3 positive cells (see Fig. 1).

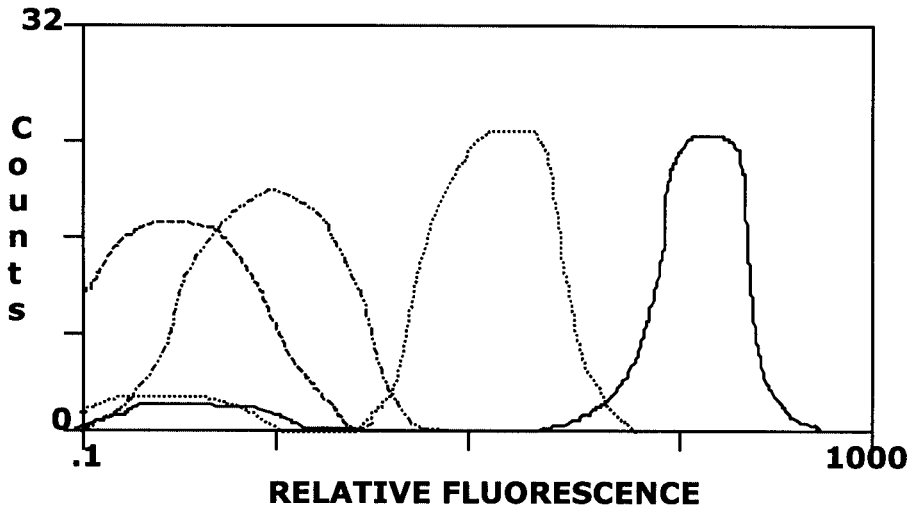


FIGURE 1. Composite drawing showing superimposed histograms demonstrating increasing fluorescence with increasing OKT3 standard concentration. Standards: ····· 0 ng/mL; ·-·-·- 50 ng/mL; ····· 300 ng/mL; ——— 600 ng/mL.

Calculations

A linear relationship between OKT3 concentration and mean fluorescence intensity can be obtained if OKT3 is limiting (not CD3 or GAM-FITC). A value for each sample is obtained by placing the cursor across the entire log histogram (e.g., channels 5 to 250 on a 256-channel histogram). The calculated mean of the cursor should be the log-to-linear converted mean. The standard concentrations are plotted on the Y-axis against the linear mean fluorescence intensity on the X-axis. The resulting standard curve should be linear or nearly linear (Fig. 2). Patient OKT3 concentrations are determined by interpolation and multiplying by any dilution factor.

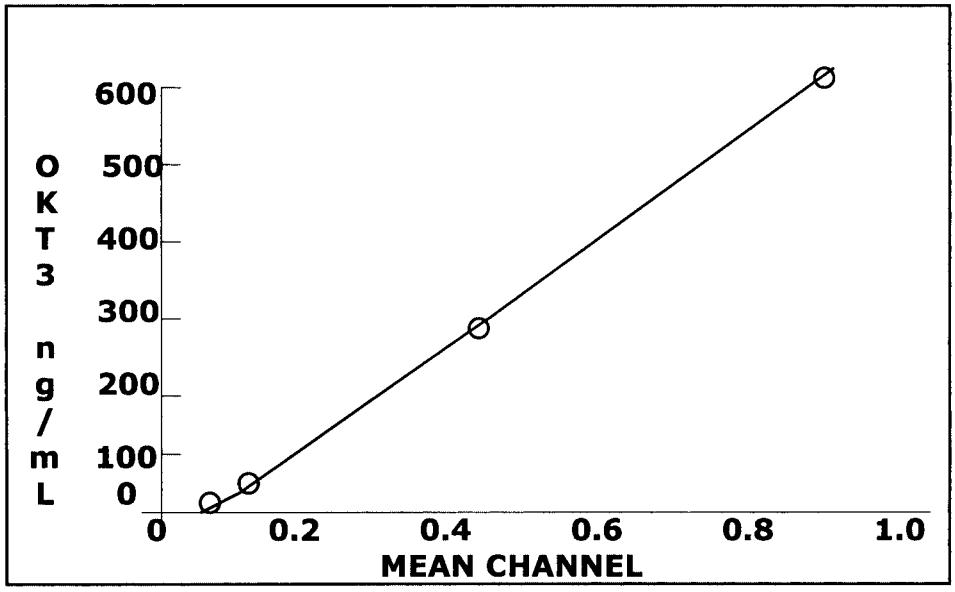


FIGURE 2: Standard curve showing typical OKT3 concentrations.

Results

Reference Range: 500 – 1500 ng/ml during steady state treatment.

Procedure Notes

When using the recommended 5 mg dosage of OKT3 for immunosuppression, plasma levels usually rise within 2 – 3 days to a steady state between 500 – 1500 ng/ml. Values are often lower during the first few days of therapy. Early abnormal consumption of OKT3 by host anti-OKT3 antibodies can be detected by a drop in plasma OKT3.

Limitations of Procedure

This assay is not designed to detect OKT3 plasma levels of < 50 ng/ml.

References

1. Goldstein G, Fuccello AJ, Norman DJ., Shield CF, Colvin RB, and Cosimi AB. OKT3 monoclonal antibody plasma levels during therapy and the subsequent development of host antibodies to OKT3. *Transplantation* 42:507, 1986.
2. Schroeder TJ, Weiss MA, Smith RD, Stephens GW. The efficacy of OKT3 in vascular rejection. *Transplantation* 51(2):312-5, 1991.
3. Wittwer CT, Knape WA, Bristow MR, Gilbert EM, Renlund DG, O'Connell JB and dewitt CW. The quantitative flow cytometric plasma OKT3 assay: its potential application in cardiac transplantation. *Transplantation* 47(3):533-535, 1989.

The Quality Assurance / Improvement Program

Deborah Crowe

Overview

The QA/QI program is established in the laboratory to ensure quality in testing for all phases of pre-analytical, analytical, and post-analytical procedures. The laboratory must have a written protocol which addresses how quality will be assessed and monitored for each of these areas. The JCAHO reference data has defined ten basic steps involved in QA monitoring and evaluation:

1. Assign Responsibility
2. Delineate Scope of Care
3. Identify Important Aspects of Care
4. Identify Indicators of Quality
5. Establish Thresholds for Evaluation
6. Collect and Organize Data
7. Evaluate Care
8. Take Action to Solve Problems
9. Assess the Actions and Document Improvement
10. Communicate Relevant Information to the Organization-Wide QA Program

A. Assign Responsibility

The Laboratory Director has overall responsibility for the Quality Assurance Program. However, to ensure quality, the Director must rely on key laboratory personnel to help implement and monitor compliance to QA policies. The QA manual should indicate all key personnel and the responsibilities assigned to each in evaluating and monitoring the indicators for quality. A Quality Assurance Committee will be needed to review QA reports on a quarterly basis and to evaluate the effectiveness of corrective actions.

1. QA Committee – Director, Lab Manager, Supervisors, department representatives.
   a. Evaluate QA needs
   b. Write general QA policies
   c. Monitor QA indicators
   d. Review corrective actions
   e. Assess effectiveness of corrective actions
   f. Present summary of QA report to entire staff
2. Lab Supervisors / Director
   a. Write specific departmental QA policies
   b. Determine QA indicators to be monitored
   c. Compile data from QA indicators
   d. Prepare Quarterly QA report for the department
   e. Review Reagent QC and Maintenance logs periodically
   f. Provide proper training for new employees and documentation of training for new methodologies
3. Laboratory Staff
   a. Document all problems as they occur
   b. Report accurate and timely results
   c. Identify and correct reporting problems
   d. Performance of quality control as required for each procedure
4. Laboratory Director
   a. Review all proficiency testing before submission
   b. Review all proficiency test results when received
   c. Determine appropriate corrective actions when needed
   d. Review Quarterly and Annual QA summary reports.
   e. Ensure that all aspects of the QA program are functioning as intended.
   f. Ensure employee competence

Each department should provide a list of the tests performed and the clinical use for the test. This will provide the basis for identifying the most important indicators of quality that will be monitored as part of the QA program.

B. Identify Important Aspects of Care

Each department must identify the areas most prone to problems and those most likely to adversely affect accuracy of testing or patient care. For example, proper collection, quality testing practices, and good communication of results to the transplant team may be important aspects.

C. Identify Indicators of Quality

For each important aspect of care, a well-defined and measurable indicator of quality must be identified. For example, for the proper collection of sample, one might monitor the number of rejected samples and the reasons for rejection. For quality testing, one might want to monitor QC failures, reagent problems, equipment problems, and performance on proficiency testing. The indicators should be objective and should help direct attention to potential problems or opportunities for improvement. A partial list of the CLIA '88 quality assurance monitors include:

- Test requisition data for completeness
- Test requisition data for relevance and necessity
- Appropriateness of criteria for specimen rejection
- The use of specimen rejection criteria
- The completeness, usefulness and accuracy of test report information necessary for the interpretation of test results.
- The ability to interpret of test results
- The timeliness of reporting test results
- Guidelines for prioritization of tests
- The accuracy and reliability of test reporting systems
- Record storage and retrieval systems
- Quality control remedial actions for effectiveness
- Corrective actions taken for errors detected in reported results
- Corrective action taken for control problems
- The effectiveness of corrective actions taken for any unacceptable proficiency testing result
- The accuracy and reliability of test systems not included in approved proficiency testing programs
- Patient test results that are inconsistent with existing clinical and laboratory data
- The effectiveness of policies and procedures for assuring employee competency
- Documentation of problems and complaints

D. Establish Thresholds for Evaluation

For each indicator, a threshold is established at which intensive evaluation of the problem is triggered. The threshold established is usually dependent on the number of tests or samples being handled. Some critical indicators may warrant 100% compliance and QA review of the variance will result from any failure.

E. Collect and Organize Data

Forms are useful to document problems, corrective actions, proficiency test misses, etc. Some labs are going a step further by entering the information into a database. This allows one to easily sort and monitor the types of problems encountered each quarter. Appropriate staff should be identified to collect the data needed for the QA report. Data should be organized so that can be easily evaluated and compared to the established thresholds for compliance.

F. Evaluate Care

For a laboratory, this refers to the quality of testing which may affect patient care. The QA committee reviews the compiled data and determines if there are any trends or patterns that may indicate a possible problem area. For example, one might see a larger number of amended reports or rejected samples compared to last quarter. An increase in turnaround time may indicate the need for additional personnel or may be related to equipment problems documented for this quarter.

G. Take Action to Solve Problem

When the threshold for a quality indicator is exceeded, members of the QA committee should examine the problem and determine if appropriate corrective actions have been taken. They should attempt to identify the cause of the problem and to provide insight or suggestions for improvement.

H. Assess the Actions and Document Improvement

The effectiveness of the corrective actions must also be monitored. If improvement is not evident by the next quarterly report, additional corrective actions must be implemented.

I. Communicate Relevant Information to the Organization-Wide Quality Assurance Program

Findings from and conclusions of monitoring and evaluation, including actions taken to solve problems and improve care, should be documented and reported through the established channels of communication. The QA summary report should be made available to all staff members and discussed at Lab meetings.

The Quality Assurance Manual

The laboratory's Quality Assurance manual should give general guidelines for maintaining quality in laboratory testing. The manual can serve as a means to organize in one place much of the information required for accreditation. The different aspects of the laboratory QA program are grouped as Pre-Analytical, Analytical, or Post-Analytical Components. Each important aspect of laboratory performance is identified and the following information is specified for each:

Goal
QA indicators
How indicator will be monitored
Evaluation – threshold for compliance and follow-up actions

The QA Manual contains the general policies for how the different components of the QA program are to be carried out. The more specific procedures and data collected are usually kept elsewhere (ex. Reagent QC, Maintenance Records,

Procedure manual). The QA manual indicates how the laboratory is to monitor QA issues. The following outline includes the major components that should be included in a QA Program.

A. Pre-Analytical

1. General Laboratory
   a. Organizational Chart – responsible persons
   b. Plan for Director Coverage
   c. Emergency Notification Plan
   d. Description of Laboratory Space
   e. List of Services Provided and Turnaround times
   f. Accreditations and Licensures
2. Personnel
   a. Job Descriptions
   b. Employee Orientation Program
      1. Risk Management Policies
      2. Disaster Plan
      3. Infectious Control and TB plan
      4. MSDS / Chemical Hygiene Plan
      5. Safety Issues and Universal Precautions
      6. Personal protective Equipment (PPE)
      7. HIV Post-Exposure Prophylaxis (PEP) Program
      8. Drug Testing policy
   c. Employee Training Program
      1. Training provided for job requirements per job description, safety, computer, personal development, and quality.
      2. Documentation of training steps
         - Read procedure in SOP
         - Watch procedure by trained technologist
         - Perform with supervision
         - Perform alone
         - Final approval by Director / Technical Supervisor
         - Documentation of training and competence
   d. Personnel Evaluation
      1. Performance Appraisal
         - Initially assessed after six months and annually thereafter.
         - Based on job accountabilities, responsibilities, goals and pre-defined measures
      2. Competency Assessment – annually
         - Direct observation of test performance
         - Monitoring the recording and reporting of results
         - Review of worksheets and QC records
         - Performance on internal and external proficiency
         - Performance of maintenance and function checks
         - Assessment of problem solving skills
         - Re-training initiated when indicated
      3. Continuing Education
         - Staff development provided to meet individual needs, regulatory and accreditation requirements, and the changing needs of the laboratory
         - Documentation of continuing education is maintained.
   e. Personnel Files
      1. Documents contained in Personnel File
         - Resume
         - Documentation of Education and/or Training
         - Licenses
         - Copy of Certifications (ex. CHT, CHS)
         - Signed Job Description
         - Signed Orientation Checklist
         - Performance Appraisals
         - Competency Checks
         - Incident reports
         - Technical Upgrades
         - Documentation of Continuing Education
      2. Review files annually to document that they contain all required forms. Check that licenses, certifications, performance appraisals, competency checks, CEUs, etc. are up-to-date.

3. Sample Acquisition – must have written criteria
   a. Sample requirements
      1. All samples must be individually labeled with patient's name or other unique identification number and date drawn.
      2. A test requisition should accompany the sample. If not, testing may begin with an oral order from the physician, but a requisition must be received within 48 hours.
      3. The specimen integrity must be preserved (ex. transit time not too long, proper temperature maintained, excessive hemolysis avoided, etc.).
      4. There must be sufficient quantity of sample for the assay.
      5. There must be compliance with proper specimen collection (correct tube, temperature, etc.).
   b. Requisition requirements
      1. The requisition should include:
         Patient ID
         Name and facility of requesting physician
         Date of specimen collection
         Time of collection, if pertinent to test
         Source of specimen
      2. The name and number on the sample vial must match that on the Request form.
      3. The Requisition should contain pertinent medical history, if available.
   c. Shipping requirements
      1. Packing instructions
      2. Storage conditions
      3. Transit time required

B. Analytical

1. Procedure Manual
   a. Policy for Review of Procedure Manual
      - Must be reviewed annually by Director
      - Recommended that testing personnel review annually and participate in updating
   b. Policy for Updating the Procedure Manual
      - Structure to link policies and procedures
        If there is a policy to have a written protocol, then the protocol must appear in the procedure manual.
      - Process to ensure uniformity of SOP and forms
        Control of document versions and effective dates
        – Utilize footer for name of procedure, version date, and page number
        – Use History of Method form to document changes to procedure and date change was made. Should be signed by Director and kept at the end of each procedure. The latest date on this form should correlate with the date in the footer of the procedure.
      - Archive old procedures
        – Remove old procedures or pages which have been changed. Write Date retired or replaced on procedure.
        – Keep old procedures for a minimum of 2 years.
   c. Validation of New Procedures
      All new procedures or modification to procedures must be validated by performing parallel studies or optimization studies. (see Quality Assurance, Chapter 2)
2. Quality Control Program (see Quality Assurance, Chapter 3)
   a. QC protocols for test methods
   b. Reagent QC
   c. Equipment Maintenance
      - Calibration and preventive maintenance in accordance with manufacturer's recommendations, regulatory requirements, and accreditation standards.
      - Complete documentation of equipment identity, results of scheduled calibrations, actions taken, and disposition of equipment is maintained.
      - Defective equipment is identified, controlled, and repaired or replaced.
3. Proficiency Testing
   a. Internal Proficiency – Tech-to-Tech comparisons
   b. External Proficiency
   c. Designation of testing personnel
   d. Review of Proficiency testing and corrective action
   e. Comparison of testing done at different testing sites
   f. Comparison of testing done by different methodologies
4. Review of Results – Correlation with Patient Information
   The laboratory must have a system in place to verify results and to ensure that the results obtained correlate with known patient information.
5. Specimen Referral
   The laboratory must list approved laboratories used for specimen referral. Copies of the accreditation of send-out labs must be kept on file. Any problems with the send-out lab must be documented.

C. Post-Analytical

1. Reporting Results – need written policy for each of the following
   a. Required Information – sample date, test date, lab #, name, results, reference range, interpretation
   b. Generation of Reports
   c. Verification of Reports
   d. Amended Reports
2. Records
   a. Storage of Records – written policies needed
   b. Confidentiality Statement
      Written confidentiality statement
      List of authorized individuals to whom results may be given over the phone
3. Policy for handling of discrepant results
   a. Discrepancies between laboratories
   b. Discrepancies between methodologies
4. Interaction with the Transplant Program and other Clients
5. Quality Improvement
   a. Review and Update of Policies
   b. Problem Identification and Corrective Actions
   c. Evaluation Thresholds
   d. Effectiveness of Corrective Actions
   e. External Inspections
   f. Communication with Staff

QA Forms

The laboratory must maintain a mechanism to document and investigate events which have a potential to affect quality or safety. Forms are very important to document QA problems and corrective actions. Each quarter, the forms are collected, sorted, and the information is recorded on the QA report. The following types of forms may be used to document problems and variances in the laboratory. Samples are included at the end of this section.

A. Problem Resolution Form

This form should be used to document any problem, no matter how minor or serious. It can be used to document problems within the lab, with a client, with the transplant program, OPO, etc. The use of these forms should be encouraged and should become part of the laboratory's routine practice. This form is used to document specimen problems, processing problems, QC problems, computer problems, or client complaints.

B. Incident Report

This form is used for more serious problems that could have been avoided if the laboratory polices had been followed. These reports must have corrective actions documented. Depending on the nature of the problem, a copy of the incident report may be placed in an employee's personnel file.

C. Equipment Failure Report

This report form is used to document instrument malfunctions and corrective actions and/or repairs.

D. Amend Report

This form is used to document that a report was changed. The reasons for the change are explained and corrective actions (if needed) are documented.

E. Proficiency Testing Corrective Action

This form is used to document misses on external proficiency testing. The results are re-evaluated and the possible problem is described with appropriate corrective actions.

The QA Report

The laboratory must maintain documentation of all quality assurance activities, including problems identified and corrective actions taken. A QA report provides a summary of all QA activity and provides a way to detect problems or trends that need further consideration. An accurate and comprehensive QA Report is vital to keeping both the Director and the Staff informed of potential problems so that a concerted effort can be made to solve them.

A major emphasis of current quality assurance standards is that the QA program be designed to effectively evaluate the QA policies and compliance with the policies. Revision of policies and procedures may be warranted based upon the results of the evaluations.

A. Frequency of QA Reporting

At least quarterly, data should be compiled on a QA report. Most problems and incidents should already be documented and on file. An example of a QA report is found at the end of this section, but many similar formats may be used. The results should be made available to the entire staff and is usually discussed at a lab meeting.

B. Safety Inspection

Part of the Risk Management Program requires that routine safety inspections be performed. These are usually done each month and included with the QA report.

C. QA Committee

All problems are reviewed by the QA committee and assessed for need for follow-up actions. Often, it may be difficult to determine if the corrective action was appropriate and the QA committee may want to re-address the problem at the next meeting to verify that the corrective action was effective in solving the problem. If not, additional corrective actions may be needed.

1. It is recommended that a log events be maintained to ensure that the proper steps in resolving a problem are taken.
2. Results of current assessments are compared to previous results.
3. Trend analysis of incidents, errors, and accidents is performed to aid in prioritizing process improvement efforts.
4. Follow-up is performed to determine effectiveness of corrective actions.

Process Improvement – Utilization of QA Data

Purpose: To define a process that can objectively measure the laboratory's level of performance, identify areas where performance can be improved, provide information that will help set priorities for improvement, offer ideas for improvement, and determine whether corrective actions actually resulted in improvement.

Procedure:

1. The Supervisor collects the QA data and summarizes the information on the quarterly QA report.
2. The QI Committee reviews the summary and looks for any trends in the data when compared to last quarter results.
3. The QI Committee will prioritize the problems that require follow-up action.
4. The QI Committee will present findings to appropriate Supervisor who will develop a team to address the problem.
5. An action plan is developed and a designated team member will implement the plan and collect data.
6. The results are reported to the QI committee.
7. The QI committee analyzes the results and determines if the improvement action was successful.
8. If the action was successful, policies and/or procedures are updated to implement the action as standard procedure.
9. If the action was unsuccessful in promoting a positive result, another action plan is developed and the process is repeated.

Benefits of a Good Quality Assurance Program

1. Provides a means whereby all members of the Laboratory from Director to Technologist can have a clear understanding of how the laboratory is performing and can identify problem areas.
2. Provides objective evaluation of problems which can be presented to management to support need for additional staff, new equipment, etc. to correct the problems.
3. The information can be used to further improve the operation of the laboratory.
4. The information can be used when discussing problems with clients. For example, if 90% of rejected samples came from one client, then this could be used to discuss the problem with that client to convince them to try to solve the problem on their end.
5. A strong QA program is essential in protecting the laboratory from legal implications of poor quality in testing. When litigation occurs, the laboratory must have adequate documentation of all actions and problems that may affect testing quality.
6. QA information may help management address issues regarding problem personnel. Proper documentation in personnel appraisals, competency checks, and incident reports are essential in protecting the laboratory if an employee is dismissed for poor performance.

References

1. DCI Risk Management and QA Program, Nashville, TN.
2. Standards for Histocompatibility Testing; American Society for Histocompatibility and Immunogenetics; March 1994.
3. CLIA '88 – Clinical Laboratory Improvement Act; Federal Register 57(40):70001, 1992
4. DCI Laboratory Policy Manual; Nashville, TN
5. LSU Medical Center- Shreveport; QA Manual
6. Bowman-Gray HLA Quality Assurance Program
7. ASHI Laboratory Manual, 3nd Edition. 1994. Ed. A. Nikaein. Ch. VI. Quality Controls
8. Metz, SJ. Quality Assurance in the Histocompatibility Laboratory. In Tissue Typing Reference Manual. Southeastern Organ Procurement Foundation (SEOPF). Richmond, 1993: Ch C.31 20-1 to 21-14.

PROBLEM RESOLUTION FORM

Quality Assurance, Assessment, Control and Improvement Program

Date:

Type of Problem:

Specimen Problem	**Processing Problem**	**Quality Control Variance**
Collection	Accessioning	Controls out of range
Labeling	Sample mix-up	Reagent Problem
Shipping	Transcription error	Instrument Problem
Integrity	Lab Accident	Technical Problem
Volume	Reporting error	Other
Requisition	Interpretation error	Computer Problem
Other	Other	Client Complaint

___Specimen recollection ordered
___Sample verification required
___Test cancelled

Description of Problem:
Attach any other explanatory documents to this form

Corrective Action:

Problem reported to: **Time:** **Tech:**

Reviewed by:_____ **Date:**_____

Follow-up by Quality Assurance Officer:

Comments:

	Yes	No	N/A
Presented at QA meeting			
Needs follow-up			
Problem Corrected			
Interdepartmental notification			

Signature:_____ Date:_____

INCIDENT REPORT

Documentation of laboratory incidents that may affect safety or patient results

Date of Incident:

Nature of Incident: (Circle) Laboratory Quality Safety Client Relations
Other:

Employee Involved:

Seriousness of Incident: (circle) Serious Moderate Minor

Description of Incident:

Corrective Action: (steps taken to prevent re-occurrence)

Proper Authorities notified: _____

Reviewed by:

Supervisor _____ Date: _____

Lab Manager _____ Date: _____

Lab Director _____ Date: _____

QA Committee Review: _____ Date: _____

Laboratory Name, Address

EQUIPMENT FAILURE REPORT

Name of Instrument: _____

Manufacturer: _____

Under Warranty: ____Yes _____No **Service Contract:** _____Yes _____No

Description of Problem:

Service Required: _____Yes _____No **Cost of Repair:** _____

Corrective Actions:

Reviewed by:

Supervisor: _____ Date: _____

Lab Manager: _____ Date: _____

Director: _____ Date: _____

QA Review: _____ Date: _____

AMEND REPORT

Documentation of Error Correction

Patient Name:	_____	Lab No:	_____
Referring Facility:	_____	Department:	_____
Date Error Occurred:	_____	Person involved:	_____
Date Error Found:	_____	Error Reported by:	_____
Date Error Corrected:	_____	Corrected by:	_____
Corrected Report sent:	Yes / No	Date Sent:	_____
Authorized Person notified:	Yes / No	Person Notified:	_____

Description of Error:

Note: Attach copy of Incorrect and corrected report; Must indicate "corrected report". Keep copies for department. Send Amend form and reports to Supervisor and Lab manager for review and then to QA Coordinator for forwarding to Lab Director for review and signature.

Nature of Error: (Circle)
Serious (affected patient care) *Moderate* (could have affected patient care)

Minor (not used in patient care or correction involved update of previous result based on more information or family studies)

Corrective Action: (steps taken to prevent re-occurrence of problem)

Reviewed by:

Lab Manager: _____ Date: _____

Director: _____ Date: _____

QA Review: _____ Date: _____

Laboratory Name, Address

PROFICIENCY TEST CORRECTIVE ACTION

Survey: _____ Sample ID: _____
Date Tested: _____ Tech: _____
Consensus Result: _____
Laboratory Results: _____

Possible Problem:

Corrective Actions:

Reviewed by:

Supervisor: _____ Date: _____

Lab Manager: _____ Date: _____

Director: _____ Date: _____

QA Review: _____ Date: _____

Laboratory Name, Address

Laboratory Name
Address

QUALITY ASSURANCE REPORT

Year: _____ Quarter: 1st 2nd 3rd 4th

Date of Report:_____

Reviewed by: _____

Fire Drill: Yes / No Date:_____

Safety Inspection: Yes / No Date:_____ Tech: _____

I. Pre-Analytical

		Monthly Tally			
Indicators	**Threshold**				**Total**
Specimen Problem					
Collection Problem	<5				
Mislabeled Sample	<2				
Sample Integrity	<5				
Sample Volume	<5				
Shipping Problem	<5				
Requisition with required information (review 20 requisitions)	100%				
Misc. Problem Resolution forms (attach copies to report)	<5				

QA REPORT (page 2)

____ Quarter

II. Analytical

		Monthly Tally			
Indicators	**Threshold**				**Total**
Processing Problems					
Accessioning Problem	<2				
Sample Mix-up	0				
Transcription Error	0				
Lab Accident	<2				
Tech Error	<2				
Interpretation Error	<2				
Misc. Problem Resolution forms	<5				
Turnaround Time met (review 20 reports)	>95%				
External Proficiency (% correct)	>95%				
Internal Proficiency (% correct)	>90%				
No. of **QC** corrective actions	<2				
No. of **Reagent** corrective actions	<2				
No. of **Equipment Maintenance** corrective actions	<2				

III. Post-Analytical

Indicators	**Expected**				**Total**
Reports contain all required information (review 20 random reports)	>95%				
No. of **Amended Reports** (Attach copies)	<2				
No. of **Client Complaints** (Attach copies of report)	<2				
No. **Incident Reports** – due to noncompliance to lab policies	<2				
Computer Problems					

Comments / Follow-up

QA COMPLIANCE DOCUMENTATION

To be completed monthly by Director or Supervisor responsible for monitoring compliance to QA policies and procedures.

Prepared By: _____ Date: _____

1. Is there evidence that Problem Resolution Forms and other QA forms are being used to document variances in the laboratory?
   Yes / No

2. Have there been any Incidents due to failure to follow lab policy this month? _____

   If Yes, was there proper documentation? _____
   Corrective Action? _____
   Follow-up needed? _____

3. Has all Equipment Preventive Maintenance been performed according to schedule? _____

4. Has all reagent QC been documented and reviewed? _____

Documentation of Misc. QA policies:

Item	Frequency	Completed? Y/N	By whom?	Date
Method Comparison	January and July			
Storage of Records for 2 years; (check availability of 5 records that are 1-2 years old)	November			
Internal Proficiency – tech checks	Monthly			
Employee Competence	December			

Ideas for tasks which can be made easier/safer by changing a process or re-designing the task:

_____
_____
_____
_____

Miscellaneous Observations and Comments:

_____
_____
_____
_____

Quality Assurance of Information / Data in the Laboratory: New Test Validation, Patient Test Management, Computerization, and Laboratory Data Maintenance

Lori Dombrausky Osowski

Principle

The laboratory must incorporate a component into the Quality Assurance Program for data management issues including new test validation, patient test management, laboratory data maintenance and computerization. This chapter will discuss important issues for the laboratory personnel to maintain in order to have a viable and meaningful Quality Assurance Program, but by no means encompasses all future issues that may be identified as important to monitor. As new laboratory methods, software and new information systems become available in the future, a Quality Assurance Program must grow and mature with the technology.

I. New Test Validation

All new and revised tests must be validated prior to implementation by the laboratory. All new procedures must be approved by the Director / Technical Supervisor. All technologists performing the new procedure must document that they have been trained and are competent to perform the new or modified procedure. The following components must be completed and approved:

A. Parallel Testing

1. Parallel testing is performed on materials with another validated method or with another accredited laboratory performing the same test.
2. A blind study is preferred, in which the laboratory is unaware of the results of the test samples prior to testing.
3. The amount of parallel testing needed may be designated by an accrediting agency or determined by the lab itself, depending on the amount of new testing that is being brought into the laboratory.

B. Reproducibility Studies

1. Intra-run reproducibility – documentation that the same answer is obtained when the sample is tested in duplicate on the same run.
2. Inter-run reproducibility – documentation that the same answer is obtained when a sample is tested on two different runs.

C. SOP (Standard Operating Procedure)

The new procedure must be written and incorporated into the department's procedure manual. Old procedures that have been replaced with a modified version must be removed from the procedure manual. The date the old procedure was retired or replaced is written on the old procedure and the old version is saved for two years.

D. Documentation of Training

1. A training guide (if necessary) is established.
   If this is a new procedure, a module may need to be added to the training manual to incorporate the tasks necessary to master in order to perform the new procedure.
2. Documentation of training of all personnel who will be performing the new procedure.
3. Quality control, equipment calibration and maintenance must be established and implemented.
   a. Proper QC measures must be established for the procedure and these must be included in the SOP for the procedure.
   b. QC forms may be needed for the procedure for proper documentation that adequate QC was performed and was within tolerance limits set for the procedure.
   c. Equipment calibration procedures must be included in the SOP and must be documented to have been performed. Tolerance limits for accepting or rejecting calibration results must be established.

d. Preventive maintenance procedures for equipment used in the test must be established and included in the SOP or Equipment Maintenance manual. Forms may be needed to document that preventive maintenance was done according to the schedule established in the laboratory.
e. The impact of any internal and external operations must be assessed. For example, if incubation conditions are changed, one must validate the effect of the change on test results after proper parallel studies have been performed.
f. After the new test is in place and is operating as an SOP, then the process must be monitored at intervals to determine if the new test is effective as implemented to attain the laboratory's initial goal.
g. Flow charts or checklists may also be helpful to help aid in this process (see Figure1).

Figure 1.

Test Validation Checklist		
TASK	**BY**	**DATE**
1. Design a validation protocol		
2. Construct a flowchart of the process		
3. Perform Parallel Testing		
4. Write an SOP		
5. Write a training document		
6. Formulate Competency Training Forms		
7. Determine necessary equipment and reagent quality control		
8. Write a quality control SOP		
9. Design QC forms to capture QC data		
10. Determine the necessary preventive maintenance and calibration schedule for equipment		
11. Design a training schedule for the new SOP		
12. Train personnel and document training		
13. Assess effect on internal and external operation processes		
14. Assign or develop any needed system checks		
15. Collect data on the quality indicators (system checks) and monitor performance		
16. Implement any necessary corrective action		
17. Conduct any necessary process improvement activities		
18. Design forms needed to capture any results from the new SOPs		

II. Patient Test Management

Patient test management involves setting protocols and monitoring compliance for patient preparation, specimen collection, labeling of samples, transport of samples, and processing of samples during testing. The laboratory must have written procedures for each of these and all tests must be accompanied by a written request within 30 days.

A. Test Requisitions – must include following:

1. Patient name or other unique identifier
2. Name and address or other suitable identifier for requesting client
3. Tests ordered
4. Date of specimen collection
5. Other relevant information – ex. ethnic group, relationship to recipient, immunization events, drugs
6. Oral requests must be followed by a written request within 30 days.
7. Requisitions must be kept for a minimum of 2 years.

B. Patient Preparation

1. Instructions for special preparation of patient must made available to the client for each test performed in the laboratory. For example, serum for crossmatch must be drawn prior to dialysis, at least 2 weeks after a sensitizing event, or prior to induction immunosuppression.
2. There should be a policy for when pre-scheduling is required for a test. This information needs to be made available to client.

C. Specimen Collection

1. The lab shall have written criteria for sample volume, type of anticoagulant, storage conditions, and transport requirements for each test ordered.
2. The samples for testing in the HLA laboratory must be collected in the appropriate tubes and stored under the correct conditions in order to maintain cell viability during transport and storage.

D. Labeling of Samples

1. The sample must be properly labeled with name and/or identification number and the date drawn. The initials of the phlebotomist should also be on the tube.
2. Criteria for rejecting samples:
   a. Sample unlabeled
   b. Identification of tube and requisition do not match
   c. Poor viability due to improper storage and/or transport
   d. Incorrect tube used for collection
   e. Insufficient quantity to perform test
   f. Tube broken

E. Transport of Specimens

1. Sample tubes must be shipped in special specimen mailing boxes, which are double-lined, and include protective packing to prevent breakage during shipping.
2. A biohazard label must be attached prior to shipping.

F. Processing of Specimens

1. Ensuring Reliable Specimen Identification during Processing
   a. Samples must be properly labeled and match requisition
   b. The sample is given a unique laboratory accessioning number which is used during processing.
   c. The laboratory number is placed on all worksheets and tubes used during testing.
   d. When reading trays, the number appearing on the worksheet and tray are re-checked and matched before recording results.
2. Relationship of Patient Information to Patient Test Results
   a. The results are reviewed by at least two individuals
   b. The results are compared to past results and family typing to ensure that they do not conflict with previous data.
3. Turnaround time is monitored to ensure that results are reported in a timely fashion.
4. Clients must be notified of test changes that affect test outcome or interpretation. SOPs must reflect these changes.
5. There must a mechanism in place to monitor complaints and problems that affect patient test management and clinical consultation available to clients. (See Figure 2)

Figure 2.

Patient Test Management Checklist

TASK	BY	DATE
1. Does the laboratory must have written procedures for patient preparations, specimen collection, labeling and transport?		
2. Are all tests accompanied by a written request within 30 days?		
3. Do test requisitions include: the patient name or other unique identifier, name and address or other suitable identifier for requesting client, the tests to be performed, date of specimen collection, and any additional data relevant and necessary to a specific test, in order to assure timely testing and reporting of results, such as ethnic group, relationship to other family members, immunizing events or drugs?		
4. Are requisitions kept for a minimum of two years?		
5. Are turnaround times monitored to ensure timely reporting of results to clients?		
6. Is a list of test methods, performance specifications and other data that may affect interpretation of results available to clients?		
7. Are clients notified of test changes that affect test outcome or interpretation? Do SOP's reflect changes?		
8. Is there a mechanism in place to monitor complaints and problems that affect patient test management? Is clinical consultation available to clients?		
9. Has an SOP been written for patient test management issues? Is there a written protocol for sample handling during the testing process to ensure that proper identity is maintained?		
10. Are personnel trained properly and training documented?		
11. Assess effect on internal and external operation processes		
12. Assign or develop any needed system checks		
13. Collect data on the quality indicators (system checks) and monitor performance		
14. Implement any necessary corrective action		
15. Conduct any necessary process improvement activities		
16. Design forms needed to capture any results from the new SOPs		

III. Computer Validation Protocol

The laboratory must perform validation and revalidation of computer systems including the associated software, whenever new hardware, new or upgraded software, and new and changed interfaces are implemented.

A. Validation of New Computer Programs

New computer programs must be documented and verified to perform as expected and validated for accuracy after installation.

B. Monitoring of Accuracy of Computer-Assisted Calculations or Interpretations

All applications that perform an analysis that was previously performed manually must be monitored for accuracy on a regular basis to assure correct performance.

C. Dedicated Personnel for Computer Program Maintenance and Upgrades

Access to the computerized systems must be limited to appropriate persons, in order to maintain integrity, security and confidentiality of data.

D. Computer Back-up Systems

There must be tracking capabilities of electronic records and activities, a protocol for backing up data, ability to reissue data electronically and a backup plan for "down time" incidents.

E. Computer Support

Support services for the system must be identified and in place. (See Figure 3)

Figure 3.

Computer Systems Validation Checklist

TASK	BY	DATE
1. Design a validation protocol		
2. Construct a flowchart of the process		
3 Have new programs been documented and verified to perform as expected and validated for accuracy after installation?		
4. Write an SOP		
5. Write a training document		
6. Formulate Competency Training Forms		
7. Is access to the computerized systems limited to appropriate persons in order to maintain integrity, security and confidentiality of data?		
8. Is there a tracking capability for electronic records and activities?		
9 Is there a protocol for backing up data, ability to reissue data electronically and a backup plan for "down time" incidents?		
10. Are there support services for the system identified and in place?		
11. Design QC forms to capture QC data		
12. Design a training schedule for the new SOP		
13. Train personnel and document training		
14. Assess effect on internal and external operation processes		
15. Assign or develop any needed system checks		
16. Collect data on the quality indicators (system checks) and monitor performance		
17. Implement any necessary corrective action		
18. Conduct any necessary process improvement activities		
19. Design forms needed to capture any results from the new SOPs		

IV. Laboratory Data Maintenance

The laboratory must maintain data storage and maintenance, have appropriate access to data, and verify data for accuracy.

A. Laboratory Records

1. The test reports must be delivered promptly to the authorized person(s) .
2. Duplicates of reports should be maintained by the laboratory for minimum two years.
3. Data must be reported in a timely, reliable and confidential manner.
4. Test records must specify the condition and disposition of specimens that do not meet the laboratory's established criteria for specimen acceptability.

5. Requirements for reports
   a. Testing laboratory's name,
   b. Testing laboratory's address
   c. Pertinent test and normal values
   d. Collection date of sample
   e. The unique sample identifier number
   f. Name of individual tested
   g. Date of report
   h. Test results
   i. Test methods (when appropriate)
   j. Appropriate interpretations
   k. Signature of Lab Director or Designee
6. Panic values must be called directly to clients.
7. The lab must maintain confidentiality and security of data.

B. Storage of Records

1. The lab must follow regulations regarding long term storage of records and documents.
2. Records must be kept and readily available for at least two years, but may be longer, depending on which regulatory agencies oversee the laboratory. (See Figure 4)

Figure 4.

Laboratory Data Maintenance

TASK	BY	DATE
1. Maintenance, have appropriate access to data, and verify data for accuracy		
2. Are the test reports delivered promptly to the authorized person(s) and are duplicates of reports maintained by the laboratory for minimum two years?		
3. Does data reported in a timely, reliable and confidential manner?		
4. Do test records specify the condition and disposition of specimens that do not meet the laboratory's established criteria for specimen acceptability?		
5. Does the report must include the testing laboratory's name, address and pertinent test and normal values? Are panic values directly delivered to clients?		
6. Do reports contain: the collection date of sample, the lab's unique identifier, name of individual tested, date of report, test results, test methods and appropriate interpretations and signature of the lab director, or designee?		
7. Does the lab maintain confidentiality and security of data and follow regulations regarding long term storage of records and documents? This time is at least two years, but may be longer, depending on which regulatory agencies oversee the laboratory.		
8. Design an SOP		
9. Train personnel and document training		
10. Assess effect on internal and external operation processes		
11. Assign or develop any needed system checks		
12. Collect data on the quality indicators (system checks) and monitor performance		
13. Implement any necessary corrective action		
14. Conduct any necessary process improvement activities		

References

1. B, *A Model Quality System for the Transfusion Service*, Transfusion Service Quality Assurance Committee, 1997.
2. *Clinical Laboratory Improvement Amendments of 1988, final rule*. Federal Register, 57(40):7001,1992.
3. Cox, F., S. Vaidya and G. Teresi, *Quality Assurance for Serology and Cellular Methods*, ASHI Laboratory Manual, 3rd Edition. VI.9.1
4. ASHI Accreditation Standards, Guidelines and Checklist, March 15,1995.

Facilities and Environment

Geoffrey A. Land

Overview

An integral part of any Quality Assurance or Continued Quality Improvement Program is the assessing of workplace safety. There are several regulatory agencies that routinely monitor laboratory working conditions (HCFA, CDC, JCAHO, OSHA). Moreover, they have determined that employees have a right to know about what hazards or potential hazards will be encountered while performing their jobs and that they must receive this information in their initial training. These agencies further require management to develop action plans to resolve any physical or environmental problems in the workplace, implement the plan, and document the success of their actions by thorough review of the data. Finally, the employee's knowledge of the information must be documented through performance evaluations and competency tests.

To have a viable laboratory safety program, it is not enough to have written policies and procedures. It is necessary to apply these policies and procedures in a consistent evaluative process. This process includes, but is not limited to, the consistent collection of and supervisory review of all environmental data (ambient and testing temperatures, hazardous chemical and biological exposure, etc.). More importantly, values outside defined acceptable ranges must be brought to a supervisor's attention immediately and corrective action must be taken and documented. Results of environmental assessments made by other than laboratory personnel (electrical safety, fire safety, air handling, etc.) must not only be available for review by regulatory agencies and the institution's administration but also for review by the laboratory staff. As with all laboratory documents, environmental assessments should be readily accessible and it is suggested that these materials be collated into a single electronic or paper file/folder.

This chapter describes the various categories of environmental factors to be assessed, specific items within each category, and required or recommended practices for dealing with specific hazards. The factors and their degree of relevant importance or risk will vary among laboratories and over time within a laboratory. As laboratory practices and methods change, so may the environmental hazards, rendering this chapter incomplete. No rules or guidelines can substitute for a commitment to assuring a safe work place.

I. Physical Facilities

A. Space

1. **ASHI Standard C1.000**. *(UNOS C1.100): "Laboratory space must be sufficient so that all procedures can be carried out without crowding to the extent that errors may result."*
   Federal Regulation 493.1204: *The laboratory must provide the space and environmental conditions necessary for conducting the services offered.*

   With that said, there are no hard and fast rules about the amount of space necessary to accomplish all of the tasks implicit in histocompatibility testing and the assurance of quality results. However, inadequate space may cause a variety of serious problems including:
   a. Jostling a nearby worker, causing a spill of hazardous materials
   b. Specimen mix-ups
   c. Sub-optimal test performance
   d. Increased injury risk
   e. Violation of federal, state, and/or local regulations
   f. Demoralization of technical staff and reduced attention to detail
2. A space of approximately 30 square feet per individual is usually adequate for a single task. This space accommodates a 5 ft. x 2 ft. bench, 1 ft. clearance, and a 3 ft. wide aisle. The three feet aisle provides unobstructed space for anyone working behind the individual at this space. However, additional space is necessary for:
   a. test equipment (e.g., microscopes, centrifuges, biosafety hoods, fume hoods, incubators, thermocyclers, computers, water baths);
   b. storage of specimens and reagents at required temperatures;
   c. record storage that provides easy access;
   d. segregation of certain functions (e.g. pre- and post- DNA amplification, specimen handling and paperwork) and certain types of hazardous materials (e.g. radioisotopes, materials that produce toxic fumes, etc.);
   e. storage and disposal of hazardous materials (e.g. human tissues, sharps, radioactive waste, combustibles, etc.);
   f. appropriate numbers and types of safety equipment (e.g., fire extinguishers, eyewash stations, safety showers, fire blankets, hazardous spill kits, etc.); and
   g. storage of personal protective equipment.

B. Extent of Service

1. Lighting must be sufficient to prevent eye fatigue, especially for those tasks requiring pipetting small volumes.
2. Ventilation must be adequate to prevent accumulate of potentially toxic gases (e.g., CO_2, N_2, etc.) and/or volatile toxic chemicals.
3. Facility and equipment temperature verification
   a. Ambient temperature and humidity must be controlled within the range specified for optimal test performance. The ambient temperature must be monitored on a daily basis.
   b. All temperature maintaining equipment (incubators, freezers, refrigerators, water baths, heating blocks, dry baths, thermocyclers, etc.) must be operated at temperatures optimal for their tasks or the storage of each specimen type or reagent used in the laboratory. Temperature ranges should be those defined by the laboratory's procedure manual and/or reagent manufacturer.
   c. Monitoring
      (1) Incubators, refrigerators, and freezers – daily
         (a) Recording thermometers are recommended for incubators, mechanical refrigerators, and freezers. Otherwise, manual temperatures must be recorded with linear or minimum/maximum thermometers that have been calibrated with a National Bureau of Standards thermometer.
         (b) Refrigerators and freezers – should be coupled with audible alarm, which can be heard 24 hours per day
         (c) For CO_2 incubators – temperatures and CO_2 concentration should be monitored daily. The latter should be within ± 1% of the concentration specified in the procedure manual for that task.
      (2) Thermocyclers – monthly, or as needed for discrepant reactions
      (3) Liquid nitrogen – level of LNO_2 monitored at intervals which ensures an adequate level is present at all times. An automated LNO_2 system with recording temperature and on board alarm is recommended. If a Dewar flask is used then, depending upon the rate of evaporation of that particular unit, then monitoring can be as often as daily or once or twice a week.
   d. All temperatures and gas concentrations (CO_2 and LNO_2) are recorded on a form initialed and dated daily by the recording technologist and must be reviewed by the General Supervisor and Director on a monthly basis.
4. The facility must provide for emergency power and backup freezer space, should either or both fail.

C. Mechanical Safety

1. Mechanical safety has to do with the positioning of objects so that they do not inhibit free movement of the employee.
2. Guidelines for preventing some frequent causes of laboratory injuries include:
   a. Eliminate projections that protrude into corridors and work areas (doorknobs, fire extinguishers, sharp edges and floor attachments).
   b. Provide adequate space for movable objects such as drawers, doors, and machinery to operate freely. Place guards and shields on equipment with exposed moving parts, whenever possible and provide warning labels or signs in all other cases.
   c. Supplies must not be stored in corridors and work areas. These present hazards that may cause serious falls, particularly if visibility is reduced by smoke or power failure.
   d. Dangerous reagents or heavy objects must not be stored on high shelves and at least an eighteen inch clearance must be provided between the top shelf or its contents and the ceiling (Note: This height may differ according to local fire or safety regulations).
   e. Chains or other safety strapping must be used to hold heavy tanks such as those used for compressed gases (oxygen, nitrogen, etc.) upright and pressure reducing regulators must be used to limit gas flow.
   f. If engineering or physical plant personnel monitor mechanical safety, copies of any evaluations must be made available to laboratory personnel.
3. Employees should know the locations of all safety equipment, such as spill kits for flammable solvents, fire extinguishers, fire exits, safety showers (the best method of extinguishing burning clothing), and fire blankets, in addition to the person(s) to call when the general safety of the workplace is compromised.

D. Electrical Safety

All employees should have general knowledge of the fundamental principles of electricity and electrical safety. This should include a general understanding of the physiology of electric shock, especially emphasizing how tetany is induced in muscle and how to avoid the electrical current running to ground through the heart. Employees need to know that electricity finds the path of least resistance to ground which, in some instances, may be through the employee's body. They should also understand the importance of grounding equipment properly, avoid overloading electrical outlets, and avoid the use of extension cords.

1. Laboratory electrical hazards represent the combined possibilities of shock, fire, and the release of asphyxiating vapors and gases. For this reason alone, there has to be an ongoing electrical safety program for the facility and its equipment.
   a. The institution's engineering or physical plant personnel usually monitor electrical safety, but it is incumbent on the laboratory staff to be aware of their findings. Consequently, copies of all documents pertaining to electrical safety must be available to the laboratory.
   b. At the least one employee per shift must know the location of the electrical control (panel) box for the laboratory and how to cut off the power supply in an emergency.

2. Suggestions for greatly reducing electrical risks.
   a. *Grounding of appliances* – accounts for most electrical accidents.
      (1) Three-prong plugs – connecting all appliances to outlets.
      (2) Ground fault circuit interrupters – useful with some appliances. They interrupt power in short circuits and can be used to test to see if the grounding circuits of an appliance are satisfactory.
      (3) Do not handle electrical items with wet hands, gloves, feet, or body.
      (4) Initially touch electrical appliances with the back of the hand, otherwise a shock may cause the fingers to curl forward and possibly prolong the contact.
      (5) Computers and other complex equipment using microchips and/or central processing units (flow cytometers, ELISA recording spectrophotometers, thermocyclers, etc.) should be plugged into surge protectors to protect them from power fluctuations. Surge protectors with battery back-ups provide uninterrupted power to crucial pieces of equipment such as computers and CPU driven equipment such as flow cytometers, spectrophotometers, thermocyclers, etc.
   b. *Preventive equipment maintenance* – identifies most developing hazards.
      (1) Examine instruments each time they are used to ensure there is no cord damage and that any necessarily exposed contacts are properly guarded.
      (2) Equipment should be monitored carefully for overheating and should be located far away from combustible materials, if routinely operated at high temperatures.
3. Hospital based laboratories
   a. All electrical equipment associated with a hospital, including hospital based laboratories, must be safety tested in accordance with the *American National Standards Institute/National Fire Protection Association 99 (ANSI/NFPA 99).*
      (1) The standards make no distinction between hospital, patient, or employee owned equipment, all must be safety tested.
      (2) Must be tested at least annually and those records must be on file and reviewed by the management staff.
      (3) All staff responsible for reporting defective equipment.
      (4) Usually done by hospital Biomedical Engineering Department. Records must be made available to the laboratory.
   b. These standards are enforced by the following agencies
      (1) The Joint Commission on Accreditation of Health Care Organizations (JCAHO)
      (2) OSHA
      (3) HCFA
   c. Medical Devices Act – requires that any medical device contributing to serious injury and/or death must be reported to the Food and Drug Administration within 10 days of the incident.
   d. Any occurrence or incident report generated by faulty electrical equipment must be reviewed by the Director and Supervisor and corrective action documented.

E. Fire Safety

1. Three ingredients are needed for fire or combustion: fuel, ignition and oxygen. The opportunities for these factors to combine in the laboratory are more frequent than one would like. Even partial burning which develops only smoke can be as dangerous as fire.
2. The precautions given below limit the fuel, ignition or oxygen sources, and will prevent or reduce the likelihood of having a laboratory fire.
   a. Fuels
      (1) Flammable liquids, such as acetone, xylene or toluene, are a primary source of fuel. (Ignition of a spill from breaking a one-gallon glass bottle can produce ceiling temperatures of nearly 900 degrees F within one minute.)
      (2) Suggested handling
         - Store relatively small amounts in safety containers (no glass) on bench
         - Keep away from electrical heating units
         - Store containers in special air-tight cabinets
         - Limit total quantity on hand.
         - Store only in refrigerators made for flammables.
         - Provide ventilation for flammable gas or vapor.
   b. Ignition sources
      (1) Static electricity or improperly grounded electrical equipment. There is enough static electricity to ignite vapors generated by simply pouring large quantities of solvents from one container to another.
         (a) Prevented by using grounded metal funnel located in a fume hood
         (b) Decreasing the distance through which the solvent is poured, and by keeping at least 50% humidity in areas of solvent transfer.
      (2) Portable heating devices, such as Bunsen burners or propane torches, are also sources that must be controlled, primarily by keeping them away from vapor areas or combustible materials.

c. Oxygen is always present where people work, but concentrated sources are found in oxidizing chemicals, such as nitric or sulfuric acid. Small amounts of fuel or a spark or small flame in the presence of an oxidizer can cause an explosion. Such chemicals should be protected by using bottle carriers and special storage areas.

3. Fire protection measures should include detection systems, employee fire drills, and clearly posted evacuation routes.
   a. The most frequent causes of laboratory fires are carelessness, lack of knowledge, smoking, unattended operations, faulty electrical devices and unsafe environments.
   b. Escape routes must be posted, as required by inspecting agencies and common sense.
   c. Precautions that must be in documented operation
      - Escape route posted
      - Outside assembly area identified for lab
      - Smoke alarm active
      - Alarm system audible
      - Sprinkler system turned on
      - Fire communication procedure identified
      - Drill practices held yearly
      - Escape route uncluttered (60-inch corridors minimum)
      - Emergency lighting available
      - Know when, where, and how to fight a fire
   d. Most of the activities encompassed within Fire Safety are usually the responsibility of physical plant personnel acting in concert with the local fire authorities. Any documents generated during these activities must be available to the laboratory.

F. Thermal Hazards

Thermal hazards include cryogenic solids and fluids, such as dry ice (CO_2), liquid nitrogen (LNO_2), and freon as well as normally functioning gas or electrically heated equipment that can cause skin burns.

1. Technologists working with LNO_2 should use face shields to avoid splashes or projectiles of broken containers that are caused by rapid warming of the LNO_2.
2. Controls for high temperature equipment should be located to avoid contact with the heating source.
3. Suggested precautions for the handling of dry ice
   a. Packaging must prevent pressure build-up by releasing CO_2 gas.
   b. Dry ice weight should appear on the outside of the package
   c. Dry ice must be placed within the secondary packaging.
   d. Secondary packaging must remain unaltered after release of CO_2
   e. Packaging must be able to withstand the temperatures and pressures encountered during transportation, if such were lost.

G. Waste Management

1. All material contaminated with blood must be bagged and labeled as biohazardous waste, and either sterilized before general disposal, incinerated or disposed in accordance with institutional, local, and state policies.
2. Containers must be leakproof and/or contain sufficient absorbent material to contain liquids so that no spills occur.
3. Blood-contaminated sharp instruments and needles must be disposed in containers that can be handled without danger of skin puncture.
4. Final disposition of medical waste must be according to local, state, and Federal regulations.

H. Hazardous Materials Program

The laboratory's Q/A program must also include documentation of adequate and appropriate management of hazardous materials. This includes proper classification, labeling, transportation, and instructions for shipping instructions of hazardous materials as well as reporting of all incidents and accidents incurred during the handling of such agents. The staff must review all documents pertaining to these materials annually.

1. Classes of hazardous materials
   a. Explosives
   b. Gases
   c. Flammable liquids
   d. Flammable solids
   e. Oxidizers
   f. Poisonous materials
   g. Infectious substances
   h. Radioactive materials
   i. Corrosive materials
   j. Dry Ice and other miscellaneous reagents/supplies

   NOTE: Transportation of materials, Chemical Hazards, and Radiation Safety will be discussed in separate sections below.

2. Labeling
   a. **Biohazard wastes:** Transport in containers with BIOHAZARD symbols printed or affixed to them. Commercial trucks are placarded according to the Department of Transportation (DOT) regulations.
   b. **Other hazardous materials:** Must have proper hazard labels placed next to the shipping name on the container. The package must accommodate all labels without having a label wrap around the package face.
   NOTE: Infectious substances (class 6.2) should have the label Class 6, *"Infectious Substance"*
   c. Diagnostic specimens: Requires the OSHA BIOHAZARD label and the following text:
   "Diagnostic specimens – packaged in compliance with IATA Packing Instruction 650."
   Diagnostic samples do not need a DOT label.
   d. All packages: Must conform to OSHA's blood borne pathogen standard for labeling.
3. Information necessary for hazardous exposure program (Chemical and Radiation exposure will be handled in separate sections. See below)
   a. Documentation of all work related accidents, injuries, and illness due to exposure
   b. Problem Resolution or Incident Reports
   c. Follow-up testing (viral serologies, culture, etc.)
   d. HIV considerations:
      (1) Post-exposure detection and prophylaxis program
      (2) Employee counseling
      (3) Permission slip to have putative source(s) tested
   e. Workman's compensation policies relative to exposure
   f. Short and long-term disability expectations
   g. Early return to work program
   h. Medical Leave Act/Disabilities Act policies as they relate to exposure
4. As part of part of any continuing quality assessment program there should be routine, documented monthly safety hazard checks as well as compliance with other departmental Q/A policies. Some items may need no more than an annual review. If these data are collected by another department, they must be made available to the laboratory

I. Transportation of Samples

1. Biological specimens must be packaged in sturdy containers with sufficient surrounding absorbent cushioning material to contain any leakage and double bagged where appropriate.
2. Fully processed blood products have generally been exempted from these requirements, being deemed by the Food and Drug Administration (FDA) as regulated products carrying little or no risk to handlers.
3. The packaging requirements for transporting untested blood products outside of the manufacturer's control requires the use of leak-proof packaging and sufficient absorbent material to contain any leakage.

   NOTE: It is the senders' responsibility to protect the shipper.
   a. Substances must be classified for shipping as described below.
      - Proper shipping name
      - Hazard class – assign only 1 (and subdivision, where applicable)
      - Identification number (see Hazards Material Table)
      - **UN** number – United Nations number, domestic and overseas shipping
      - **NA** number – North American number, US and Canada only
      - Packing group
        – Group I (great danger)
        – Group II (medium danger)
        – Group III (minor danger)
   b. Shipping Papers
      - Name and address of consignee
      - Name and phone number of responsible party
      - Nature and quantity of goods
      - Shipping name, hazard class, Packing group, UN/NA identification number, Packing instruction number
        NOTE: Infectious material have no packing group
      - Quantity of shipment by weight or volume
      - Number of packages and type
      - Indicate overpacking
      - Emergency response information – CDC emergency phone number, if material infectious
      - Name, title, place, date, and signature of person preparing package
      - Shipper's certification
        " I hereby declare that the contents of this consignment are fully and accurately described above by the proper shipping name, and are classified, packaged, marked, and labeled/placarded, and are in all respects in the proper condition for transportation according to the applicable international government regulations"
      - Diagnostic and dry ice shipments aren't restricted and require no shipper's declaration
      - For infectious substances, include under *"Additional Handling Instructions"*:
        Prior arrangements as required by the IATA Dangerous Goods regulations 1/3/3/1 have been made.

4. Material should be completely labeled and contents of package fully disclosed, as in the following examples:
   a. *Infectious substances*
      - Obtain manufacturer's Department of Transportation certification with performance oriented packaging (POP) criteria
      - Special markings necessary for infectious substance packaging
        – "UN" packing symbol
        – Packing type code = 4G
        – Text = Class 6.2/Yr of Mfg.
        – State or country international vehicle code authorizing Mfr. to ship
        – Name of manufacturer
      - Criteria for secondary packaging
        – Non-leak
        – Internal pressure ≤ 13.8 lb/in^2
        – Temperature range -400° C to 550° C
      - Itemized contents list/requisitions between inner and outer containers
      - Shipper's name and telephone number on outside package
   b. *Diagnostic samples*
      - Inner packaging
        – Non-leak
        – Secondary packaging (water tight)
        – Absorbent material between primary and secondary packaging
      - Outer packaging
        – Strength adequate for intended use
        – Withstand 1.2 meter drop and pressure tests
        – 4" Minimum dimension for shipping
      - Packing list/requisitions between primary and secondary container
      - Air shipping must be indicated on package and waybill
      - Labeling
        – Infectious substance, affecting humans
        – "Dry Ice" (when applicable)
        – UN or NA identification number
        – Name and address of consignee and consignor
        – Arrows indication correct "Up" position
        – Name and telephone number responsible party
        – Outer label: "Inner packages comply to prescribed specifications"
        – Total amount of infectious substance (e.g. ≤ 1ml)
        – Information written in English

II. Biologic and Chemical Hazards

A. Biologic Hazards

1. Job description and risk of exposure
   One of the mandates of the Occupational Health and Safety Administration Act (CFR 1910.1000 to end, 1 July, 1997) is to define the relative risk of an employee becoming exposed to a biohazardous agent based upon his/her job description.

   Category 1: Procedures and tasks relative to this job description involve exposure to blood, tissues or body fluids (amniotic fluid, pericardial fluid, peritoneal fluid, pleural fluid, synovial fluid, cerebrospinal fluid, semen, and vaginal secretions) or body fluids grossly contaminated with blood via mucous membrane or skin contact or trauma. Persons in this category are must routinely use personal protective equipment (PPE) such as disposable laboratory coats, gloves, goggles and/or face shields, while performing their tasks.

   Category 2: Routinely does not perform tasks that would lead to exposure to contaminated fluids and tissues but, upon occasion may be asked to perform Category 1 activities. Must wear PPE when engaging in Category 1 tasks.

   Category 3: Routine work does not involve any potential for association with contaminated fluids or tissues nor would they be called upon to do so in an emergency. PPE is not required for the performance of their duties.
2. Universal Precautions
   Due to the potential risks for infection in any given blood, body fluid, or tissue specimen by such agents as the Hepatitis B and C viruses and the HIV virus, it is imperative for all laboratorians to be aware the correct manner in which to process these specimens. Thus, the basic tenant of Universal Precautions is that,

   All blood, body fluid, and tissue specimens must be considered contaminated with an infectious agent.

Such a global view of infection potential has led to the adoption of more stringent measures when handling and processing specimens:

- No smoking in the laboratory
- No eating or drinking in the laboratory
- No storing of food in the laboratory
- No mouth pipetting
- No application of cosmetics
- Use of PPE (gloves, lab coats/gowns, goggles, face shields, etc.)
- Remove of PPE's when leaving the laboratory
- Wash hands with soap and water prior to leaving laboratory
- All items used within a biohazardous area are presumed contaminated (telephones, keyboards, camera, centrifuge, etc.)
- Place needles, blades, and all other sharp objects in heavy leak-proof boxes
- Discard blood and containers to autoclave or incinerator in separate biohazard trash bins.
- Contain aerosol formation when opening capped tubes, blending, sonication or mixing by using a biologic safety hood (Class I or Class II)
- Keep work area and instruments clean and neat. This can be accomplished by wiping surfaces with 0.5% (1:10 dilution) of sodium hypochlorite (bleach) prepared daily or other suitable antibacterial and virocidal disinfectant.
- Avoid wearing sandals, loose clothing, loose jewelry, neckties, and long hair styles (unless tied back or contained)

3. Portals of entry and prominent infectious agents
   a. *Fecal-oral:* primarily Hepatitis A virus (HAV): rarely occurs in the laboratory and then, usually as a consequence of improper handling of patient material. This infection is even more rare in the histocompatibility laboratory, where the majority of specimens handled are tissue or blood. This infection can be avoided entirely by the use of common sense, universal precautions, and soap and water.
   b. *Needlesticks and other "sharps" exposure:* the greatest exposure risk for viral hepatitis and the Human Immunodeficiency Virus (HIV) in the laboratory today. Needle-sticks, glassware/other sharps cuts, or problems arising during venipunctures account for the vast majority of the total number exposure incidents in any health care institution. And, because of the constant association with whole blood and the isolation of lymphocytes, the histocompatibility laboratory is exceptionally vulnerable.
      (1) Exposure guidelines that are established for one's own institution should be prominently displayed in the Quality Assurance Manual.
      (2) Guidelines should include all local, state, and federal recommendations for prevention, surveillance, and monitoring for adherence with Universal Precautions.
         (a) Surveillance must include all needlesticks, cuts, human bites and any other injury that breaks the integrity of skin or mucous membrane and places the involved employee(s) at risk of infection.
         (b) All incidents involving needlesticks and other sharps must be reported according to each respective institution's guidelines and at least a copy of any report generated during an incident must remain in the laboratory.
         (c) All incident reports must show evidence of Director review and follow-up counseling with the employee.
   c. Most common infective agents associated with blood/body fluid/tissue exposure
      (1) Hepatitis B Virus (HBV) – long incubation hepatitis; classic serum hepatitis
         (a) Portal of entry
            - In the U.S. the major mode of HBV transmission is sexual, both homosexual and heterosexual.
            - The parenteral route (entry into the body by a route other than the gastrointestinal tract) transmission , i.e., by shared needles among intravenous drug abusers and to a lesser extent in needlestick injuries or other exposures of health-care professionals to blood, tissue, or body fluids is just as important.
            - Workers are at risk of HBV infection to the extent they are exposed to blood and other body fluids. Employment without that exposure, even in a hospital, carries no greater risk than that for the general population.
         (b) Infection risk controlled mainly through administering vaccine to all employees that have a Category I or II job description. Adequate, cost-effective tests are available to evaluate post exposure immune status.
         (c) Post exposure treatment
            - *Patient originally using needle cannot be identified:* Baseline serology testing done, the puncture victim treated with immune globulin, vaccine may be administered, and immune status checked after 1 and 6 months.
            - *Needle from known hepatitis carrier:* Baseline serology testing done, several doses of hepatitis B immune globulin are routinely given, and the victim's immune status is checked after 1, 6 and 12 months.

(2) Hepatitis C Virus (HCV) – most prominent human fluid and tissue exposure risk today.
   (a) There is no vaccine available for protection and the currently available tests are costly and require molecular capabilities.
   (b) Treatment: Long term interferon
(3) Human Immunodeficiency Virus (HIV) – A very serious concern to health care workers, such that the increasing risk of AIDS transmitted via HIV demands that all precautions must be taken to prevent sharps types of injuries or abrasion and open wound types of exposure.
   (a) Primary transmission of HIV similar to HBV, although it does not occur with as high a frequency as HBV. Exposure may be from either heterosexual or homosexual contact or as a consequence of mucous membrane or parenteral exposure, including open wound exposure to infected blood or other body fluids.
   (b) Post exposure testing is adequate and of moderate expense.
   (c) Treatment: There is neither vaccine nor any other known cure for infection. Multi-faceted and life-long therapeutic drug intervention is required to maintain infected individuals, with limited success.

d. Universal precautions as it relates to the most common blood borne agents
(1) Even though not all body fluids have been shown to transmit infection, because of the ubiquity of the above agents and the great potential for a sharps exposure to occur, all body fluids and tissues must be regarded as potentially contaminated and infectious.
(2) Both HBV and HIV appear to be incapable of penetrating intact skin, but infection may result from infectious fluids coming into contact with mucous membranes or open wounds (including dermatitis) on the skin.
(3) If a procedure involves the potential for skin contact with blood or mucous membranes, appropriate barriers to skin contact must be worn, e.g., gloves, face shields, etc.
   (a) Investigations of HBV risks associated with dental and other procedures that might produce particulates in air, e.g., centrifugation and dialysis, indicated that the particulates generated were relatively large droplets (spatter), and not true aerosols of suspended particulates that would represent a risk of inhalation exposure.
   (b) If there is the potential for splashes or spatter of blood or fluids, face shields or protective eyewear and surgical masks must be worn.
   (c) Detailed protective measures for health-care workers have been addressed by the CDC and can serve as general guides for the specific groups covered, and for the development of comparable procedures in other working environments. Federal Register/Vol. 52, No. 210/Oct '87.

4. Education and Training
a. As stated above, it is mandatory for an institution involved in the handling, processing, and testing of human clinical material to provide employees with education on the relative risks of infection. Dissemination of this information must be part of the initial training of a new employee and must be provided annually as well. Most institutions do this once a year on a global basis and have a log that is signed and dated by the employee upon finishing the initial or refresher training program. Copies of this log and any other documentation of such global training must be made available to the laboratory.
b. For those situations in which the HLA laboratory is responsible for its own biohazard exposure program, a small manual should be developed for initial training and questions concerning this material should appear on initial competency assessment examinations during the early stages of employment. Thereafter, the manual must be read on an annual basis and a log must be signed and dated and/or appropriate questions asked on the annual competency examination.
c. There are many references available on the subject on the relative risk of infection with human clinical material. The literature cited at the end of this chapter lists a few of the most important ones.
d. Any training program for employees on exposure to biohazards must include the following:
   - The OSHA standard for bloodborne pathogens
   - Epidemiology and symptoms of bloodborne diseases
   - Modes of transmission of bloodborne pathogens
   - Institution's Exposure Control Plan (i.e., points of the plan, lines of responsibility, plan implementation, etc.)
   - Procedures used by facility which might result in blood exposure or exposure to other potential infectious materials
   - Methods at facility used to control exposure to blood or other potentially infectious material
   - Types PPE available at facility and where located
   - Personnel to be contacted when potentially infectious blood/tissue/fluid exposure occurs.
   - Post exposure evaluation and follow-up
   - Signs and labels used at facility for potentially infectious processes or materials
   - Facility's Hepatitis B vaccine program

B. Chemical Hazards

Another part of the *"Right to Know Act,"* requires all employers to provide their employees in depth information as to the number, types, and characteristics of all chemicals that they will encounter within the scope of their job description. Additionally, all employers whose personnel are exposed to chemicals in the work place must meet the Hazard Communication Standard (HCS). In laboratories, however, a chemical hygiene plan (CHP) may be implemented which supplants the HCS. This program must have documented evidence of continuous review and oversight by an individual, the chemical hygiene officer (CHO). The CHO may be a member of the department (technologist, supervisor, director) or may operate for the entire institution. The latter is usually a member of the physical plant staff or the safety committee but, in any case, his/her name must be known to all employees.

The Federal Government realizes that each specific laboratory environment is unique. Health care laboratories vary considerably from industry, from other institutions, and even from similar laboratories. Therefore, each facility has been given the autonomy to establish and publish their own program for the use and disposition of chemicals and reagents. These local standards must, in turn, reflect the various regulatory agencies' mandate to protect employees from exposure to hazardous material and must be accessible to each and must be adhered to once implemented. Finally, there must be documented review of the CHP's implementation and the level of adherence by employees.

The Occupational Safety and Health Administration (OSHA), which oversees and ensures employee safety, has inspectors who can and will perform unannounced inspections. These inspectors measure a laboratory's compliance with their institutional plan and they have the power to levy huge fines and, in some instances, close laboratories.

ASHI inspectors also evaluate a laboratory's facilities, environment, and safety. This includes monitoring the laboratory's compliance with their own CHP. If adherence to the plan is marginal or employee training is inadequate or the environment relative to chemical hazards is unsafe to workers or may compromise patient care, the laboratory can have its accreditation revoked. Moreover, because of its deemed status with other regulatory agencies (HCFA, UNOS, JCAHO, OSHA), ASHI is compelled to notify those agencies when such incidences occur. The end result is that the laboratory may have an unannounced follow up inspection by one or more of these agencies and its activities may be severely limited or may even be closed until any deficiencies are rectified.

Because of the individual nature of CHP's, it is necessary that an institution's CHP must reflect their actual practice and not simply parrot some other plan. Blind copying of other plans will leave the laboratory open to potentially severe penalties if it does not abide by its plan, train employees to live by that plan, and monitor that they do live by that plan. Consequently, the CHP should begin with an institutional statement of philosophy. Such a statement should acknowledge the need to implement and maintain a CHP in compliance with the rules and regulations of OSHA, the Environmental Protection Agency (EPA), and state and local governments. The goals of the program are to institute, promote, and maintain a safe working environment that minimizes accidents, reduces the risk of contamination of the environment, and reduces the exposure risk of employees and visitors alike to chemical hazards.

This philosophical statement must also acknowledge the implementation of educational programs to help employees achieve these goals and to ensure proper handling of hazardous chemicals. All employees involved in developing and instituting the plan must be identified, including supervisors responsible for implementing the program, individuals on the committee responsible for developing the plan, and the head of the department whom is legally responsible for ensuring compliance.

1. Essential features of a CHP
   - All hazardous chemicals must be identified.
   - The risk of contamination of employees by hazardous chemicals (by inhalation, ingestion, or skin contact) should be reduced to a minimum.
   - Laboratory employees and employees who handle the waste streams from the laboratory are to be protected.
   - Where appropriate, exposure to these hazards must be monitored to prove that regulatory standards have been met.
   - Medical surveillance is required to limit injury in the event of employee contamination.
   - All hazardous chemicals must be prevented from contaminating the environment
   - Compliance is regulated by the EPA.
2. Hazard Determination

   NOTE: *All hazards in the department must be identified.*

   Many laboratories interpret this as meaning that a list of all hazardous chemicals must be maintained. Another approach is to maintain a list of all chemicals, reagents, and kits used or stored in the laboratory, and then identify all hazardous substances within that list. The master list may be stored in a computerized database, from which lists for individual laboratory sections may be produced.

   a. Material safety data sheets (MSDS): MSDS are required from each manufacturer of chemicals, reagents, and kits and provide the main source of information regarding chemical hazards. They are the simplest and most complete way to accumulate chemical safety data and may be kept in an organized file or notebook or even scanned into a computer (some companies even provide their MSDS on CD Roms). These files or CD-Roms provide readily available information (see list below) for training new employees and as a post exposure reference.
      - Name
      - Manufacturer

- Distributor and relevant catalog numbers
- NFPA code
- Permissible exposure limit (PEL)
- Threshold limit value (TLV)
- Chemical abstract number
- Laboratory section(s) where used/stored
- Location of Materials Safety Data Sheet (MSDS) and the date the MSDS was prepared
- Composition if it is a mixture or kit
- Upper and lower explosion limits (UEL and LEL)
- Whether the chemical is a carcinogenic, reproductive, or acute toxin
- Whether the chemical is corrosive, caustic, flammable, or radioactive and the source of this information
- If EPA regulated, under what section of the law it is regulated

(1) These documents should be kept on permanent file and updated periodically. Updates can be obtained by going out on the internet and looking for MSDS specific websites or by contacting specific manufacturer's websites.
(2) Employees must have ready access to them, in order to handle accidents and for questions from inspectors concerning the MSDS for any chemicals that they might be using.
(3) An employee also has the right to refuse to work with chemicals if they have had no training concerning the hazards involved or have not seen an appropriate MSDS.
(4) The Health Section of the MSDS contains relevant information for hazard determination. All chemicals should be regarded as hazardous until proved otherwise. The manufacturer sometimes will send a letter stating that the material they sell is not hazardous; compliance dictates that the laboratory must either have an MSDS or letter on file for each chemical stating its potential for hazard.

c. *All acute toxins, carcinogens, and reproductive toxins must be identified.* It is advisable that these lists be posted in each section. For a chemical to be recognized as a carcinogen or reproductive toxin, animal or human studies must provide evidence of an association between the chemical and development of cancer, injury to reproductive organs, or the occurrence of abortions or fetal abnormalities. This information is also found within the Health Section of the MSDS. Other sources of information are listed in the reference section.

3. Reducing the Risk of Contamination of Employees
This risk must not be underestimated; however, it may be reduced by:
- Establishing engineering controls
- Modifying employee behavior by training and mandating appropriate laboratory behavior
- Placing adequate warnings in the workplace and in procedures
- Medical surveillance and atmospheric monitoring
- Controlling hazardous chemicals in the laboratory – establishing how they should be transported, stored, and used
- Reducing the accidents by controlling spills and the amounts used.

a. *Establishing engineering controls.*
(1) Ventilation in all rooms should be measured at least annually (preferably more often) and should be adjusted to meet OSHA guidelines or the specific recommendations of reagent or instrument manufacturers. For example: copy machines, which produce ozone, are often overlooked. OSHA has established that machines producing ozone also have ventilation requirements. On average, there must be at least 4 volume air changes per hour; ideally, there should be 10.
(2) Chemical hoods and ancillary ventilation systems should be inspected annually. However, the face velocities of each hood should be checked to see that they are operating.
(3) Simple vanometers are adequate to demonstrate and document that the hood is working. These should be read at least daily if the hood is used regularly or before use if the hood is used infrequently.

b. *Modifying employee behavior.* Employee behavior is modified by establishing training programs and mandating compliance with that training.
(1) *Training Programs* – All employees who work with or are exposed to hazardous materials must be trained before working with any hazardous material, new or old, and whenever there is a change in exposure risk due to procedural or other changes. No employee should be made to work with any physical or chemical hazard for which they have not received appropriate training. Educational requirements should be matched to the job title. All employees must receive refresher safety training at least annually. Employees must sign and date log sheets when they attend these sessions and complete a quiz during each session. The sign-in sheets and quizzes are kept on file for at least three years. Any training plan should encompass the following topics:
- Overall scope of the chemical hygiene plan
- Reading the MSDS
- Selection and use of safety gear
- Spills-how to neutralize, clean up, and dispose of the waste material whether it is acid, caustic, flammable, or a volatile carcinogen

- Understanding chemical labels
- Specific instructions on the use and disposal of all hazardous material that the employee will use in the laboratory
- Carcinogenesis and Toxicology of Chemicals
- Hoods: chemical and biocontainment

(2) *Mandated Behavior.* While working in any part of the laboratory, employees are required to adhere to the following standard operating procedures (SOPs). Any breaks in the integrity of this behavior must be reported, documented, and reviewed by the management staff and the employee counseled where appropriate.

- Carcinogens, acute toxins, and reproductive toxins must be used in designated areas only.
- While working in these areas, employees must wear appropriate protective gear, which include chemical-resistant gloves, apron, and eye protection such as glasses with protective lens or face mask as minimum standard.
- Before leaving the designated area, employees must wash gloves and decontaminate protective gear (unless they are disposable) and remove the clothing. Hands must always be washed when leaving the designated area. Designated areas must be regarded as contaminated areas.
- Eye protection and gloves must be used whenever any chemical is handled or stored.
- Contact lenses must not be worn when working with hazardous chemicals, particularly organic solvents that dissolve in soft lens plastic and thus may be held in intimate contact with the eye for prolonged periods of time. If the employee has to wear contact lenses, airtight goggles must be worn.
- Chemical-resistant, waterproof aprons must be worn when handling hazardous chemicals, particularly if they are in solution or are liquid.
- No hazardous chemical must be given or loaned to unauthorized personnel or anyone who has not received appropriate safety training within the past year.
- Employees must not smell or taste chemicals.
- Food must not be stored in the laboratory, nor can employees eat, drink, smoke, or perform any other activity that brings the hand to the face. Unconscious contamination of the eyes and mouth and subsequent ingestion are major causes of contamination.
- Horseplay or any activity that might startle another employee and cause an accident must be avoided.

(3) *Placing Adequate Warnings.* All chemical and physical hazards must be identified by clear labeling, including NFPA codes. All procedures involving the use of hazardous chemicals should have appropriate safety warnings prominently displayed within the body of the written procedure, and these warnings must be highlighted (this is a College of American Pathologists [CAP] requirement, as opposed to governmental regulation). All designated chemical storage or areas where used must be clearly identified

(4) *Controlling Hazardous Chemicals*

- No hazardous chemical must be stored above five feet from the floor. Accidents can occur when employees reach above eye level for chemicals.
- Fire codes usually limit the amount of flammable materials that can be left in the laboratory. Usually, 500 ml or less of any one chemical is a good rule of thumb. Be sure to check with local and state regulations concerning minimal amounts of chemicals that can be in the laboratory.
- All stock supplies of chemicals must be stored in vented flame cabinets.
- Flammable materials must never be stored in a refrigerator or freezer unless it is explosion-proof. Many organic solvents have flash points at or below refrigerator temperatures, and electrical controls can spark and cause an explosion. Vapor build-up in the refrigerators and freezers, which are usually airtight, can cause vapor concentrations to exceed lower explosion limits.
- Toxic chemicals or flammable chemicals must not be released or stored in airtight areas such as walk-in incubators or refrigerators because of the possible build-up of toxic levels.
- Each section of the laboratory must maintain an inventory of types and volumes of chemicals stored or used within the area and there should be enough spill-absorbent material available to absorb all of the chemical in the largest container stored.
- Highly toxic chemicals must be kept in an unbreakable secondary container.
- When chemicals are carried by hand, they must be carried in a specialized container or bucket.
- Chemicals must be examined at least annually for replacement or deterioration.
- Vent vacuum pumps must be required for all chemical hoods.

c. *Medical Surveillance and Atmospheric Monitoring*

(1) If an employee is contaminated by chemicals as a result of a spill, contamination of skin, or exposure to levels of a chemical that exceed the action limit *(half the permissible exposure limit),* or the employee develops symptoms of exposure after use of the chemical, the employee must be placed under medical supervision.

(2) Atmospheric monitoring is limited to areas where there is a possibility of exceeding action limits, such as areas where xylene and formaldehyde are routinely used.

(3) All other areas must have the hazard risk assessed. This may require additional monitoring or calculation of exposure under worst-case scenarios with the following factors considered:

- The amount of chemical in use at any given time.
- Is the chemical smell present the majority of the time?
- If entire volume of chemical were spilled on the floor at one time would it exceed the action limit?
- Number of air volume changes/unit time.

d. *Protecting the Environment*
Waste control is mandated in order to avoid contamination of the environment. The EPA has the mandate to control pollution of the environment. Sometimes this agency acts through state organizations, which is the case in Texas (i.e., the Texas Water Commission). The Texas Water Commission (TWC) is concerned with chemicals that are dumped into storm sewers, because these drain directly into surface waters and seep to the aquifers. The TWC also monitors effluent from sewage treatment plants and if hazardous chemicals are discharged from a sewage-treatment plants, owners are fined. Chemicals not metabolized by microorganisms in sewage-treatment plants must not be discharged in the sanitary sewer. The local sewage-treatment facility usually has its own regulation concerning sanitary sewer disposal. The EPA publishes a list of chemicals the agency considers hazardous. This is available to the public and is a worthwhile document to have in the laboratory. Factors to consider when storing:

- Keep only one to two months supply on hand at any one time
- Recycle where possible
- No chemicals must be poured down the sink or otherwise introduced into the local sewer system unless local, state and Federal statutes permit it
- A hazardous waste tag signed by the CHO/Safety Officer and the Department Director must accompany all chemical waste picked up for transport off-site
- Only recognized disposal companies must transport and dispose of EPA-regulated substances

C. Emergency Response
As a quick reference for laboratory accidents, emergency responses are given below to limit injuries until appropriate medical care is available.

Type	Problem	Action
Electrical Safety	Burn	1. Pull the plug or cut off current. 2. Check vital signs (heart, breathing). 3. Cover burn with sterile pad or clean sheet. 4. Keep person warm. 5. Call for medical help.
Fire Safety	Flames on Person	1. Drop and roll on ground or wrap in blanket and roll. 2. Use shower or blanket. 3. Use appropriate fire extinguisher. 4. Call for medical help.
	Thermal Burn	1. Remove person form heat or remove heat from person. 2. Apply cold water to the area. 3. Check breathing. 4. Cover burn with sterile pad or clean sheet. 5. Keep person warm. 6. Call for medical help. 7. *Do not use* oils, sprays, or ointments.
Biohazard	Puncture Wound	1. Allow bleeding. Wash thoroughly with warm water and soap. 2. Report to supervisor and employee health department. Save needle/sample for testing.
	Spills	1. Wash skin with soap and water. Wash work area with appropriate disinfectant.
Chemical Hazard	Burns	1. Follow directions on MSD sheets and/or container label for treating exposure to that chemical. 2. Flush with copious amounts of cold water as per local standards and/or neutralize and flush. 3. Cover burns with sterile pad or sheet. 4. Call for medical help. 5. *Do not use* oils, sprays, or ointments.
	Volatile Liquids	1. Follow directions on MSD sheets and/or container label for treating exposure to that chemical. 2. Call for medical help.

III. Radiation Hazards

Overview
Since the last edition of this laboratory manual, there has been an even more significant decline among laboratories using radioactive material for patient testing. There are three major factors responsible for this decline: 1) The development of non-radioactive methods for detecting antigen – antibody reactions or other immunologic mechanisms at a level hitherto thought only possible with radioactive isotopes, 2) The decline in the use of the mixed lymphocyte culture as a result of increased use of molecular typing to define Class II specificity, and 3) The increasing costs of disposing radioactive wastes. However, for those laboratories that still use radioactive decay as a detection method or have the potential to begin using radioactive isotopes, the following section is intended.

A. General Guidelines

1. The quantities of radioactive materials employed in the histocompatibility laboratory and the resulting radiation levels are generally below what is required to cause immediate radiation induced illness. More concern should be placed on the long-term genetic and somatic effects, which can occur as a result of chronic low-level exposures. Since the exact dose at which genetic damage or cancer induction may occur is unknown, common sense dictates that all exposures should be kept as low as reasonably achievable (ALARA).
2. Either the Federal Government or a state agency controls licensing for all laboratories within the United States and each publishes guidelines for the safe use of radioactive materials. Further guidelines are individually determined by each licensed institution to encompass all of the radioactive protocols it is involved in. Each laboratory performing techniques requiring the use of radioactive materials must be licensed by their institution and be familiar with all governing guidelines for the safe use of such materials prior to purchase.

B. Definitions

Listed below are the definitions of a few words that are necessary in order to understand what is required of an institution in the use and disposition of radioactive materials.

1. **ALARA** – As Low As Reasonably Achievable – a commitment made by each licensed laboratory to keep all exposures as low as reasonably achievable. Federal guidelines require that all licensees determine ALARA levels and strive to achieve these levels at all times (typically one tenth of the annual regulatory limit).
2. **Activity** – The number of nuclear transformations occurring in a given quantity of material per unit time.
3. **Alpha Particle** – A charged particle emitted from the nucleus of an atom having a mass and charge equal in magnitude to those of a helium nucleus.
4. **Avalanche** – The multiplicative process in which a single charged particle accelerated by a strong electric field produces additional charged particles through collision with neutral gas molecules.
5. **Becquerel** – A unit of activity. One becquerel equals one nuclear disintegration per second.
6. **Beta Particle** – Charged particle emitted from the nucleus of an atom, with a mass and charge equal in magnitude to that of the electron.
7. **Bremsstrahlung** – Secondary photon radiation produced by deceleration of charged particles passing through matter.
8. **Curie** – The old unit of activity. One curie equals 3.7×10^{10} nuclear transformations per second.
9. **Electron** – A stable elementary particle. Constituent part of an atom often emitted during nuclear disintegration.
10. **Film Badge** – A pack of photographic film that measures radiation exposure for personnel monitoring. The badge may contain two or three films of differing sensitivity and filters to shield parts of the film from certain types of radiation.
11. **Gamma Ray** – A short wavelength electromagnetic radiation of nuclear origin (range of energy from 10 keV to 9 MeV) emitted from the nucleus.
12. **Half-Life** – The time required for a radioactive isotope to lose 50% of its activity by radioactive decay.
13. **Ionizing** – Ionizing radiation includes gamma rays, alpha rays, beta rays and neutrons (any of which can damage cellular DNA), potentially resulting in cell death, cancer or mutation if dosage is high. Conservative assumption would be that any exposure involves some risk. The severity of exposure depends upon the half-life of the radioactive source, the type of radiation, the penetrating power, the part of the body exposed and the duration of exposure. Precautionary measures with regard to use of ionizing radiation in the Histocompatibility Laboratory seek to prevent either internal or external body exposure to the material being used.
14. **Non-ionizing** – Non-ionizing radiation may include microwaves, lasers, ultraviolet light and ultrasonic sources that primarily put the eyes at risk of exposure.
15. **Radioactive Decay** – Disintegration of the nucleus of an unstable nuclide by spontaneous emission of charged particles and/or photons.
16. **Ring Dosimeter** – A monitoring badge in the form of a ring and usually containing a thermoluminescent dosimeter (TLD) chip in place of the film packet.

C. Detectors

1. *Geiger-Muller Counter*
   The Geiger-Muller Counter or GM counter is a gas-filled detector designed to detect any radiation capable of producing ionization within the tube. The GM conducting shell is filled with a gas which has a very low affinity for electrons (i.e. argon, helium or neon). A fine wire is mounted at the center connected to a positive high voltage

source. Any particle entering the tube capable of ionizing even one molecule will initiate an avalanche of ionizations and discharges in the counter that will result in collection of electrons at the center wire. The resulting charge can be measured. This counter measures all types of radiation but for some low energy emitters a thin window is required to allow penetration through the shell.

2. *Scintillation Counter*
   Scintillation counting is an ideal method for quantitating radioactivity since all forms of radiation released, alpha, beta and gamma, can be detected in very small quantities. A scintillation detector consists in its most basic form of a scintillator, a photomultiplier tube and associated circuits for counting light emissions produced by the scintillator. When a charged beta or gamma particle is released into a scintillator it imparts energy to the atoms in the scintillator, which in turn release light proportional to the energy imparted. The photomultiplier tube produces an electrical impulse when stimulated by light emitted from the scintillator, which is used to plot a spectrum for the radiation measured that distinguishes between isotopes.

D. National Radiation Council (NRC) Guidelines

All aspects concerning the production, transportation, possession, use and disposal of radioactive materials is strictly controlled by Federal, State and local authorities. It is crucial that Federal guidelines be extensively researched prior to obtaining any radioactive materials. State regulations are typically patterned after N.R.C. regulations found in the *Code of Federal Regulations, Title 10, parts 19 and 20 (10 CFR 19-20)*. This volume is available at a reasonable cost from any federal government printing office bookshop.

1. *Licensing*
   a. All laboratories anticipating the use of radioactive materials must obtain a license from the proper authorities.
   b. Different types of licenses exist for different institutions.
      (1) *Broad Scope License:* Used by large institutions for all isotopes which are used on the campus.
         - Lists all isotopes used on the campus
         - Does not detail specific procedures.
         - Controlled by a previously approved radiation safety committee within the institution. This safety committee then controls issuance of sublicenses to the individual laboratories or investigators within the institution.

      (2) *Individual license:* For laboratories that are not under the umbrella of a larger institution
         - Must submit extensive procedures
         - Designated safety officer to intercede with authorities and maintain safe operating conditions.

E. Exposure Limits

The standards for maximum permissible dose allowable for radiation workers is set by the NRC or State authorities. The current maximum exposure levels are as follows:

1. Occupational Exposure Areas *(REMS/Year; NCRP Report No. 39, 1971)*
   a. Whole body, lens of eye, red bone marrow, gonads (5)
   b. Hands and feet (75)
   c. Forearms and ankles (30)
   d. Any other specific organ not mentioned above (15)
   e. Fetus gestation period (0.5)
2. Authorities within specific governing areas or the institutional radiation safety officer may place further monthly or quarterly exposure limits.
3. The NRC and most "Agreement States" now require that each institution develop a program to maintain personnel exposures below "ALARA" limits. These limits are set by each institution. Information on specific ALARA limits can be obtained from the Radiation Safety department of each institution.

F. Required Records

1. A complete record must be kept upon receipt of an isotope until its final disposal.
   a. *Large institutions* – materials are usually received in the radiation safety department where all materials are logged in and tested for leakage upon arrival and some of the records concerning these activities or the entire tracking history of a shipment may be kept in the safety office.
   b. *Smaller institutions* – receive, log, and leak test as delivered to them. Individual laboratories are required to keep complete records of a shipments history.
2. Some of the records required are as follows:
   a. *Receipt* – Upon receipt of radioactive materials, detailed records must be filed including all receiving documents. These records must be organized in a logical manner and available for inspection at all times. Upon inspection, laboratory personnel should be able to quickly determine the exact amounts of each isotope or material that they have on hand.
   b. *Leak Testing* – Each package delivered should be tested for container integrity and possible leakage prior to storage or use. These records are often kept on specialized forms. As in all other records the leak testing records must be available for inspection at all times. In the case of large institutions where materials are received in a central location, records for leak testing may be kept in a central area. Clarification of institutional procedures should be obtained prior to licensing.

c. *Use* – Detailed records of use must be kept. Records of amounts used, employee removing, amounts remaining and disposal procedures should be logged for each use. Each laboratory should be able to trace in detail any material received from receipt to removal from laboratory.

d. *Disposal/Waste* – Most of the waste generated in a histocompatibility laboratory has very low levels of radioactivity. Radioactive waste may be generated as liquid, solid or vial form. The waste for each different nuclide should be stored and disposed of separately and according institutional, state, and Federal guidelines.
   (1) A number of different disposal options are available. The method chosen depends on the half-life of the isotope in question, the quantities generated, the concentration of the isotope in the waste and the space available for storage.
   (2) Waste storage and disposal procedures must be developed with proper authorities upon licensing.
   (3) Examples of disposal options available are as follows:
      (a) *Incineration by institution* – facility and institution must be approved prior to use
         • Effluent must be sufficiently dilute to meet requirements for concentrations found in *10 CFR 20 appendix B, Table II.*
         • Records of each incineration must be maintained.
      (b) *Burial* – waste will be packaged by institution and sent for burial in approved site.
         • As of 1993 each state is required to develop burial sites within state boundaries. Until such sites are developed burial of waste will be limited and quite expensive.
         • All institutions in states where no burial sites have been approved are required to obtain approval for onsite storage for varying periods of time.
      (c) *Decay* – Waste is generally stored for a period of time not less than 10 times the half-life of the isotope in question. The waste must then be surveyed prior to disposal.
      (d) *Sanitary Sewer* – It is permissible to dispose of liquid wastes in the sanitary sewer as long as the concentration of radioactivity is less than that considered safe for an adult to drink or breath. Federal or State guidelines should be consulted to determine permissible levels for the areas in question.

e. *Employee Exposure* – Three principal rules govern radiation safety, Time/Distance/Shielding:
   (1) **Time** – exposure is directly related to the amount of time spent in the vicinity of the isotope (i.e. decrease time by one-half and exposure will decrease by one-half)
   (2) **Distance** – the relationship between distance and exposure from a radioactive source is governed by the inverse square law. As the distance increases by a factor of two the exposure decreases by a factor of four.
   (3) **Shielding** – the type of shielding which is required for protection depends on the type and energy of the radioactive emission. Alpha particles impart their energy very quickly and do not penetrate the skin so no shielding is required. Beta particles are generally intermediate in penetrating ability and can best be blocked by acrylic shields. Gamma particle require heavy shielding such as lead or concrete. However, care should be taken to avoid lead shielding for beta emitters as beta particles will interact with lead to produce Bremsstrahlung radiation.

f. *Personnel Monitors*

   As discussed previously, all laboratories using radioactive materials are required to keep detailed records on personnel exposure. Therefore, it is necessary to obtain reliable personnel monitors for personnel working with isotopes. Two different types of monitors are generally used for this purpose, film badges and thermoluminescent dosimeters,
   (1) Film Badges

      Film badges are the most popular type of personnel monitoring device. This badge consists of photographic film sealed inside a labeled packet. The packet is mounted inside a plastic case wedged between shielding of varying types and thickness to distinguish between various energies. This packaging gives a measure of total body exposure and type of radiation. Although the film badge is sensitive, inexpensive and portable some problems do exist. The film can be sensitive to heat and of course light. It is important that the badge be cared for properly and that the package remain intact and to remember that film is not sensitive to very low energy emitters.
   (2) Thermoluminescent Dosimeters (TLD)

      TLD's can be worn as personnel monitors much like film badges. TLD badges are composed of crystalline substances whose electrons are excited to a higher state upon absorption of radiation. When these substances are heated to high temperatures the electrons return to their normal state. Upon return to their normal state energy is released in the form of light. Lithium Fluoride is commonly used used in TLD's. TLD monitors consist of lithium fluoride (or other appropriate materials) sealed inside a labeled, portable holder that can be worn in the same manner as a film badge. Advantages of the TLD are: 1) less sensitive to heat and can detect a much broader range of energies, 2) it gives a permanent record of personnel exposure and, 3) it can be annealed at very high temperatures and reused. However, that in effect destroys any permanent record of personnel exposure. The one great disadvantage of the TLD badge is that it is more expensive.

g. *Contamination/Decontamination*
Should an accident occur involving contamination to an area, immediate attention should be given to localizing the contamination and removing as many personnel as possible from the area. Specific protocols for accidental contamination should be developed by the radiation safety department of each licensed institution. It is important that prior to using radioactive materials all personnel be trained in the safety rules for their prospective institutions. Some general guidelines are listed below:
- Localize the spill to prevent spread to other areas of the lab. If aerosolization is a possibility remove personnel and seal the area.
- Check all personnel for contamination and isolate any who may be contaminated.
- Call appropriate safety personnel for guidance in decontamination. If contamination is below a certain level the lab personnel may clean the contamination up themselves. Institutional guidelines must be followed at all times.
- Decontaminate and survey to determine safety prior to return of personnel.
- Document the incident and keep on file for possible inspection by authorities.
- Should personnel be contaminated, measures to treat or decontaminate should be taken immediately. If the person requires medical attention they should be treated immediately as if the contamination does not exist. Once stabilized or if personnel do not require medical attention the following series of steps should be undertaken:
  i. Personnel must be surveyed with appropriate instruments to determine contamination.
  ii. Contaminated clothing must be removed, bagged and placed in an appropriately shielded area for decay or disposal.
  iii. Skin contamination – care should be taken to prevent spread to other areas of the body. The contaminated area should be washed extensively with a mild detergent and warm water followed by resurveying.
  iv. The procedure should be repeated as necessary until contamination is removed.
  v. Harsh detergents containing lye or hot water should be avoided. Also scrubbing if used should be gentle to avoid penetration of the skin.
  vi. If contamination cannot be removed, help should be sought from safety personnel knowledgeable in alternate decontamination procedures.
  vii. The incident and all procedures used to decontaminate the area must be documented and available to the laboratory.

h. *Employee Training* – Standard operating procedures on the processing, handling, and use of radioactive material must be written and submitted to the regulatory agencies prior to obtaining a license. It is incumbent upon the Director and Supervisor to ensure that all personnel have read these SOP's, are conversant with them, and are accurately following them in their practice. All competency examinations for employees working with radioactive material should have questions dealing with the proper handling and processing of isotopes as well as managing contamination.

i. *Licensing* (see above, D1.)

j. *Safety Surveys* – Work areas, including bench tops, floors and storage areas should be monitored frequently for removable contamination. The most common method of survey is the "wipe test," in which a known area (typically 100 cm^2 or a 10 x 10 cm square) is wiped with a cotton tipped applicator or swab soaked in detergent. The swab is then counted in a scintillation counter appropriately set for each isotope used in the laboratory. Threshold values, above which an area is considered to be contaminated, are determined by each institution. Any area found to be contaminated should be cleaned and resurveyed. All survey values before and after decontamination must be kept for inspection purposes.

3. General Rules of Conduct for personnel working in a radiation environment:
   a. The radioisotope laboratory must be used only for radioisotope work. Unnecessary materials should not be brought into the laboratory, and unnecessary work must not be done there.
   b. Work must be done rapidly but carefully.
   c. Each bottle, flask, tube, etc., which contains radioactive material must be identified by proper radiation warning labels; including amount remaining in the container.
   d. Care must be taken to avoid splashing, splattering, or spilling radioactive liquids.
   e. Smoking, eating, or drinking in the laboratory prohibited at all times.
   f. The laboratory must be kept clean and orderly at all times.
   g. Pipetting by mouth is prohibited.
   h. Absorbent paper must cover work benches, trays, and other work surfaces where radioactive materials are handled and the possibility of spillage might occur.
   i. Disposable plastic or rubber gloves must be worn while working with radioactive solutions when hand contamination is likely.
   j. When procedures are completed, monitor hands for contamination.
   k. Unshielded bottles, flasks, beakers, and other vessels that contain more than 100 mCi of activity must not be picked up by hand for more than a few seconds. Whenever practical and always when the handling time is long, tongs or forceps must be used.

l. Radioactive materials which emit gamma rays and whose activity exceeds 500 mCi must be kept behind lead shields or inside of lead lined vessels. Normally shipping containers are adequate for low level activity storage.
m. PPE must be worn as needed.

References

FACILITIES AND ENVIRONMENT

1. American Society for Histocompatibility and Immunogenetics (ASHI), January,1998. ASHI Standards for Histocompatibility Testing. Kansas City.
2. Code of Federal Regulations, July 1, 1997. Occupational Health and Safety Administration (OSHA) 1910.1000 to end. U.S. Government Printing Office, Washington.
3. Crowe, D, 1998. Quality Assurance in the HLA Laboratory. Southeastern Organ Procurement Foundation (SEOPF), Richmond.
4. Tenover, F. and McGowan, JE, 1995. Section II. Laboratory Management and Regulatory Issues. In: Murray, PR, et.al., Manual of Clinical Microbiology, 6th ed. ASM Press, Washington.
5. Transfusion Service Quality Assurance Committee, AABB, 1997. A Model Quality System for the Transfusion Service. American Association of Blood Banks (AABB), Bethesda.

EXPOSURE TO BIOHAZARDS

1. Assignment of Exposure categories – Joint Advisory Notice; Department of Labor/Department of Health and Human Services; HBV/HIV Notice. Federal Register 52 (210):91821, October 30, 1987.
2. Hepatitis
   a. Centers for Disease Control: Recommendations for protection against viral hepatitis. Morbidity and Mortality Weekly Report 34:313, 329, June 7, 1985.
   b. Centers for Disease Control: Update on Hepatitis Prevention, Morbidity and Mortality Weekly Report 36:353, June 19, 1987.
   c. Koff RS, 1995. Chapter 92. Hepatitis B and Hepatitis D. In: Gorbach SL, Bartlett JG, Blacklow NR, eds. Infectious Diseases (2nd ed.) p850 – 863, WB Saunders, Philadelphia.
3. Human Immune Deficiency Virus
   a. Center for Disease Control: Recommendations for prevention of HIV Transmission in Health-Care Settings. Morbidity and Mortality Weekly Report. 36:25, 1987.
   b. Human T-Lymphotropic Virus Type III-Lymphadenopathy Associated Virus: Agent Summary Statement. Morbidity and Mortality Weekly Report 35:540, 1986.
   c. Resnick L, Veren K, Salahuddin SZ, Tondreau S: Stability and inactivation of HTLVIII/LAV under clinical and laboratory environments. JAMA 255(14):1887, 1986.
   d. Zenilman JM, 1992. Chapter 128. Prevention of Human Immunodeficiency Virus Transmission. In: Gorbach SL, Bartlett JG, Blacklow NR, eds. *Infectious Diseases* (2nd ed.) p1169 – 1183, WB Saunders, Philadelphia.
4. Waste Management
   a. Grument FC, Macpherson JL, Hoppe PA, Smallwood LA: Summary of the Biosafety Workshop. Transfusion 28:502, 1988.
   b. Strain, BA, 1995. Chapter 7. Laboratory safety and Infectious Waste Management. In: Murray PR, Baron EJ, Pfaller MA, Tenover FC, and Yolken RH, eds. *Manual of Clinical Microbiology*. p. 75 – 85 ASM, Washington.
5. General
   a. CDC-NIH Manual. *Biosafety in Microbiological and Biomedical Laboratories.* US Dept. of Health and Human Services, Public Health Service, Center for Disease Control and National Institutes of Health, US Govt. Printing Office, 1984.
   b. Morbidity and Mortality Weekly Report, August 29, 1986.
   c. Morbidity and Mortality Weekly Report, 38(5-6): 1.
   d. Needle Sticks Take a High Toll, The Draw Sheet. University of Virginia Publications, p 30, 1981.
   e. Rose SL: *Clinical Laboratory Safety.* Chapters two, four, and five. J.B. Lippincott Company, Philadelphia, PA, 1984.
   f. Slobadien M: In: *Laboratory Safety, Theory and Practice.* Chapter three, p 60. Fuscaldo A, Erlick BJ, Hindman B, eds. Academic Press, New York, NY, 1980.
   g. Steere NV: Laboratory *Safety, Theory and Practice*, Chapter one, p 4-56. Fuscaldo AA, Erlick BJ, Hindman B, eds. Academic Press, New York, NY, 1980.

HAZARDOUS CHEMICALS

1. EPA Title III List of Lists, Document No. EPA 560/4-91-011 Section 313. Document Distribution Center, P. 0. Box 12505, Cincinnati, OH 45212.
2. NCCLS General Laboratory Practices and Safety Vol. 6, No. 15, Clinical Laboratory Hazardous Waste.
3. Federal Register Vol 55, No 21. Part 1910 of title 29 of the Code of Federal Regulation (CFR), amendment Jan. 31, 1990.
4. Annual Reports. National Toxicology Program. U.S. Department of Health and Human Services.
5. Gregory M, 1995. Chapter 1b. Microbiology Laboratory Safety. In: Mahon CR and Manuselis G, Jr. eds. *Diagnostic Microbiology* p. 32 – 48. WB Saunders, Philadelphia.

RADIATION HAZARDS

1. Noz ME, Maguire GQ Jr: Radiation Protection in the Radiologic and Health Sciences. Lea and Febiger, Philadelphia, PA, 1979.
2. Shapiro J: Radiation Protection: A Guide for Scientists and Physicians. Harvard University Press, Cambridge, MA., 1972.
3. Sorenson JA, Phelps ME: Physics in Nuclear Medicine. W.B. Saunders Co., Philadelphia, PA, 1987.
4. Radiation Regulations and Protection Procedures. Baylor University Medical Center, Revised 1989.
5. Basic Radiation Protection Criteria. NCRP Report No 39, National Council on Radiation Protection and Measurements, Washington, D.C., 1971.
6. Code of Federal Regulations, Title 10, Parts 0 to 50, Office of Federal Register National Archives and Records Administration, Washington, DC, 1988.

Quality Control Program

Anthony L. Roggero and Deborah O. Crowe

Principle and Purpose

The quality of results produced in any laboratory is only as good as the quality of the reagents used. Therefore, one of the most important functions in the laboratory is the quality control of its reagents. Quality control of reagents assures that they are functioning optimally in all tests performed by the laboratory. A reagent is any chemical or biological product used in a laboratory test. It is of critical importance that reagents are properly labeled, stored, prepared, and handled. Failure to do so may adversely affect testing results, and may also pose a threat to the health and safety of testing personnel. Quality in laboratory testing is also dependent upon the performance of equipment and instruments. Proper calibration, routine preventive maintenance, and troubleshooting must be part of the quality assurance program.

A. Components of QC Program

1. Quality Control measures and thresholds for each test performed
2. Criteria for accepting or rejecting results
3. Participation in Proficiency Testing
4. Reagent QC and tolerance limits
5. Equipment calibration and preventive maintenance
6. Documentation of problems and corrective actions

B. Quality Control Measures for Specific Test Methods

1. Each test procedure must indicate quality control measures taken and how they are used in the interpretation of the results. Procedures written in NCCLS format will usually include a Quality Control heading in which the quality control measures are detailed for ensuring that the test is performing as expected.
2. Tolerance levels must be established and corrective actions documented when controls fall outside the expected range. Worksheets used to document QC results should have the tolerance limits clearly indicated.
3. Patient results are not reported if the quality control for the test procedure exceeds the tolerance limits. There must be written criteria for accepting and rejecting results.

Proficiency Testing

1. In-House Proficiency testing – primarily used for tech-to-tech comparisons
2. External Proficiency Testing – the lab must participate in an external proficiency test for every test that is performed in the laboratory. If no commercial proficiency test is available for a test methodology, the lab should attempt to set up parallel testing with another lab that is doing the test at least every 6 months.
3. Review of Proficiency Testing – The director must review proficiency results upon completion of the testing and prior to mailing the results. The director/ technical supervisor must review the findings of the proficiency testing and document discrepancies with the consensus.
4. Corrective actions must be initiated if a result is found to be unacceptable when compared to the consensus result from other labs. Follow-up actions are important to ensure that the corrective action was effective in solving the problem.

Reagent Quality Control

A. Labeling Reagents – a written policy for labeling reagents is required.

1. All reagents must be clearly labeled for identity and concentration of the reagent.
2. The reagent label must indicate the date the reagent was prepared and/or received in the laboratory and the date it was opened or put in use (PIU).
3. The reagent must be labeled with the lot and batch number, the expiration date, and the initials of the person who prepared or opened the reagent.
4. The storage conditions should be included on the label.
5. A hazard warning label must be affixed to every reagent container indicating the type of hazard the reagent may have (e.g. toxic, etc.).

B. Vendor List

1. Establish approved vendor list to ensure vendor qualifications
2. Identify critical equipment, supplies, and services
3. Contract review
4. Establish systems to inspect, log, and store reagents

C. Inventory Control – to ensure adequate supply

1. A **Reagent List** should be kept by the laboratory (see example number 1). This list should contain the following:
   a. Name/Chemical formula where possible
   b. Hazard Category (check for MSDS)
   c. Manufacture source and Cat. #
   d. Preparation Instructions
   e. Storage Requirements
2. A **Reagent Log/Quality Control Record** must be kept to record information on new batches or lots of reagent. (See Examples 2 and 3)
   The log should contain the:
   a. Name of reagent
   b. Lot number
   c. Date received
   d. Expiration date
   e. Date prepared or put in use
   f. Initials of the person preparing or opening the reagent quality control results
3. All reagents MUST be discarded upon expiration.
4. Mixing of reagents between different lots of commercial kits is usually not permitted since the test was validated at the factory using the combination of reagent lots found in the kit.
5. **Reagent Inventory** should be done on a regular basis to ensure adequate supply. For each reagent, one should establish the optimal order size, the lead time needed for ordering, and the quantity remaining when a new order should be made.

D. Documentation of Reagent Performance

1. Every reagent has minimal standards it must meet before it can be placed into use (e.g. pH, support of cell viability, sterility, etc.).
2. Any new reagent lot must be checked for acceptable performance parameters prior to being placed into use to ensure that it is of the same quality and meets the same standards of performance as the reagent currently in use.
3. Any deviation from acceptable ranges necessitates repeat testing and/or consultation with the laboratory Supervisor and/or Director.

E. Procedures for Reagent QC must be written with detailed instructions. Forms should be available to record and document QC performance. The forms must include the tolerance limits for acceptable performance and documentation of review. The procedures should be found in a Reagent QC section of the SOP or in the Reagent QC manual.

1. **Titration Procedures** – for Complement, AHG, and monoclonal antibodies [ex. Monoclonal Antibodies (CD2/CD20, PE) or FITC-anti-IgG reagent for crossmatch]
2. **Parallel Testing** – for typing reagents, magnetic beads, separation media, etc. The old lot and new lot are both tested with the same sample.
3. **Documentation of Acceptable Performance** and "**Date in Use**" for reagents used as "components" in another reagent. This may include such reagents as magnesium chloride, dNTPs, NaCl, etc. For these reagents, one may simply indicate in separate columns on the Reagent Log/QC sheet the date when the reagent was first used and if the test performance was acceptable (see Example 3)
4. **Protein Supplements** (ex. AB serum or FCS) – requires Cytotoxicity testing prior to use. Most easily done by placing on serum screening test
5. **Negative Control** (Normal Human Serum – NHS) – should be tested for Cytotoxicity. Most easily done by placing on serum screening test.
6. **Positive Control** (Anti-Lymphocyte Sera) – Cytotoxicity testing and titer of positive control should be determined – titer should not be higher than seen commonly with alloantisera.
7. **Ability to Support Cell Growth** – Media, Fetal Calf Serum, etc. is checked for its ability to support cell growth for the length of time required by the procedure for which it will be used (ex. MLC).
8. **Special Notes** on the use of some reagents should be included in the procedure for which they are used.

F. Rejection Criteria for Reagents

1. New lots of reagents that significantly differ from expected test results.
2. New lots of reagents that significantly differ from the parallel or previous control test results.
3. Reagents that alter test results.
4. Sterile reagents that failed sterility check.
5. Expired reagents that failed re-quality control testing.
6. Sterile reagents that have not been opened under sterile conditions (under a biological laminar flow hood).
7. Any contaminated bottles must be discarded.

G. Storage Requirements

Reagent Sera	≤ -20°C (≤ -70°C recommended)
Patient Sera	≤ -20°C (≤ -70°C recommended)
Typing Trays:	≤ -70°C to -80°C
PRA trays	≤-70°C to -80°C required;(-135°C/LN_2 recom.)
Complement	≤ -70°C to -80°C
Cells in DMSO	≤ -70°C to -80°C
Tissue Culture reagents	4°C
Immunomagnetic Beads	4°C
Antibiotics	-20°C

Some reagents require special testing prior to use in order to determine the purity, toxicity, or optimum reactivity (titration assays) of the reagent. Of particular concern to the lymphocytotoxicity assay is complement and anti-human globulin reagent quality control. For DNA typing, the most extensive reagent QC is done with Primers and Probes. For Flow Cytometry, the FITC-conjugated anti-IgG reagent requires the most care when determining the optimal working dilution. Because of the complexity of these reagent checks, a brief protocol for each is given below.

Complement QC

All new lots of complement should be tested in parallel with old lots or with defined cell samples on at least 5 tissue typing trays.

"Checkerboard" testing (using dilutions of the new lot of complement vs. dilutions of known antisera) should be performed to determine the strength and toxicity of any new lots of complement (see example 3 for Complement "Checkerboard" form).

Expiration dates for complement and anti-human globulin should be assigned either one year from the date of quality control completion or use the manufacturer's expiration date – whichever is the longer dating. Expired complement and anti-human globulin can undergo re-quality control testing and upon acceptance have the expiration date extended for one year. Any lot that fails re-quality control testing must be discarded.

A. PROCEDURE: New Lot of Complement Evaluation

1. Choose two well-characterized antisera.
2. Choose three well-characterized cells: two that will give positive reactions with the antisera and one that will give negative reactions.
3. Antisera should be used neat (1:1), 1:2 through 1:16. Dilutions can be made with negative (AB) serum.
4. Each dilution is tested with the complement at different dilutions and also with no complement (Complement control or spontaneous lysis control).
5. Complement should be used neat (1:1), 1:2, 1:4, 1:8 and 1:16. Dilutions can be made with appropriate diluent such as RPMI, barbitol buffer, etc.
6. It is essential that new and old lots of complement be tested simultaneously.
7. Positive and negative controls need to be included with each cell tested.
8. A possible tray layout for setting up this complement evaluation, can be found at the end of this chapter.
9. From this study, the complement lot with the best reactivity is chosen. This new lot of complement then needs to be evaluated for use with the laboratory's different test procedures (NIH, AHG, etc.) as well as with different target cells (PBL, B cell, etc.). The complement is tested in parallel with the different crossmatch techniques and with a DR tray to document that it performs satisfactorily under all conditions for use.
10. Care should be taken not to continually reduce the strength of a new lot of complement chosen. This will lead to poorly defined reactions over time, under previously similar test conditions.

B. Special Notes on Complement

1. Complement is heat labile. Long-term storage of complement must be at -65°C or colder.
2. Complement should be kept cold when dispensing aliquots for refreezing. Use an ice bath if aliquoting large quantities.
3. Complement reactivity is destroyed by heating at 56°C for 30 minutes.
4. Gentle mixing when thawing will reduce damage to complement proteins.
5. Violent mixing can cause premature activation.
6. Chelating agents, such as EDTA, can deplete calcium ions necessary for the activation of complement, causing false negative reactivity.

Anti-Human Globulin (AHG) QC

Similar to Complement QC, all new lots of anti-human globulin (AHG) should be tested in parallel with old lots or with defined cell samples.

A strongly positive serum, a serum that reacts with a specified antigen and, if possible, a weak serum that reacts only in the presence of AHG should be used in a "Checkerboard" testing similar to Complement QC (using dilutions of the new lot of AHG vs. dilutions of known antisera). Titers are compared to determine the strength and toxicity for any new lots of AHG.

The AHG titration must include defined cells with and without the antigen for which the serum has specificity. (see example 4 for Anti-Human Globulin "Checkerboard" form).

A. Procedure for AHG Evaluation

1. Choose several well-characterized complement-dependent antisera for testing. These should include a strongly positive serum that reacts with a specified antigen and, if possible, a weak serum that reacts only in the presence of AHG.
2. Choose well-characterized target cells that will react with the antisera selected above.
3. Take a 72 well microtiter tray and dispense 1 µl of the dilutions of one antisera across the tray. Column A on the tray (12 wells) will contain the antisera neat (1:1). Column B will contain the antisera at 1:2, etc.. Column F will contain the negative control.
4. Add 1 µl of a chosen cell preparation to the entire tray. Incubate 30 minutes at room temperature.
5. Wash the tray 3X.
6. Add dilutions of antiglobulin reagent (make reagent and dilutions just prior to use; keep all dilutions cool, 2-6ºC), from the weakest dilution (bottom of tray) to the strongest dilution (top of tray). One dilution is dispensed across an entire row of wells. Row 12 will have a dilution of 1:180 of the antiglobulin dispensed into it and Row 4 will have a dilution of 1:20. Rows 1-3 should not have any antiglobulin reagent dispensed into it.
7. The antiglobulin reagent should only be allowed to sit in the wells for 1-2 minutes prior to adding 5 µl of complement to each well.
8. Incubate the trays an additional 60 minutes at room temperature.
9. Stain cells and record reactions.
10. A possible tray layout for setting up and recording this anti-human globulin reagent evaluation can be found at the end of this Chapter.
11. The optimal dilution of antiglobulin reagent is that which gives 90-100% cell death with the highest dilution of antisera, and highest dilution of antiglobulin reagent. There may be two or three wells (or dilutions) of reagent that demonstrate this maximum efficiency.
12. The optimal dilution of antiglobulin reagent for any cell/serum combination should give at least a two-fold increase in titer strength above that titer observed with the NIH method. Example: If the NIH method gives an "8" (80%+ cell death) at a dilution of antisera of 1:2, the antiglobulin reagent (one or more dilutions) should demonstrate an "8" with a titer at least of 1:8 or greater.
13. Combining the results seen with the different cell/serum combinations, it is possible to choose a dilution of the antiglobulin reagent that will work satisfactorily with most cell/serum combinations.
14. Choose an AHG reagent that has an optimal working dilution of at least 1:16. One that works at 1:64 to 1:256 will allow the laboratory to conserve reagent and preclude the necessity of frequently having to evaluate antiglobulin reagent.
15. Dispense small aliquots of reagent and store at -70°C. Pull a tube, thaw and dilute (with RPMI) the reagent to the appropriate working dilution just prior to use.
    Note: If the AHG reagent is to be used pre-mixed with the complement, the titration should be done in a similar manner. The range of titers used should be approximately 6X that used in the above to account for the "final" concentration of AHG used in the test (1 µl working dilution of AHG + 5 µl of Complement).
    Example: When AHG is titered as described above, start with a 1:20 and go to 1:180. If pre-mixed with Complement, the dilutions tested should include 1:120 to 1:1080 in its range.

B. Monthly Complement and AHG Quality Control

1. On a microtiter tray, dispense a negative control (AB serum) in duplicate.
2. Add a known antiserum in dilutions from neat (1:1) through 1:64 (or higher, depending on titer of antiserum). The same control should be used each month. Dispense the serum dilutions in duplicate. Multiple QC trays may be made and stored at -70ºC for future use.
3. Add a previously prepared cell prep to the quality control tray. The cell chosen must contain the antigen for which the antiserum is specific.
4. Perform the test using the NIH and AHG procedures.
5. Record the titer strength of reactivity. This will be the highest dilution of serum that gives a "6" or "8" reaction.
6. A reduction in titer over time indicates that a new lot of complement needs to be put in use.
7. The titer with the AHG method should be at least 2 dilutions greater than that seen with the NIH method.

Primer QC for DNA Typing

A. New Primer Set QC for SSP Methods

1. Positive Reference DNA Panel
   A panel of reference DNA can be constructed in a set of tubes that parallels the SSP panel to be tested. For example, if the first tube in the SSP panel is specific for DRB1*01, then the first tube in the reference DNA panel contains DNA that has the DRB1*01 gene. Each tube in the DNA panel will be positive with the corresponding tube of the SSP template. The reference DNA may be from a homozygous typing cell (HTC) known to have the allele of the primer mix being tested. Other reference DNA may come from heterozygous individual having the allele

of the primer mix being tested. This can be from a patient that has been previously typed or from a proficiency test sample. The positive panels should show a specific band of the correct size for every well.
Construction of Reference DNA panel:

a. Identify DNA that can be used in the reference panel.
b. Divide 2 by the DNA concentration in µg/µl to determine the amount of DNA to dilute to 100 µl with *complete PCR buffer**. This will give a final concentration of 20 µg/µl.
   * For 50 ml of Complete PCR Buffer
   13.0 ml 10 X PCR Buffer
   923 µl dNTP mix (25mM)
   13.0 ml 25 mM $MgCl_2$
   23.1 ml ddH_2O
c. Place the diluted DNA/PCR buffer mixture in a Reference template that corresponds to the panel being tested.
d. Store in refrigerator or aliquot in smaller amounts and freeze.

2. The SSP panels should contain 5 µl of the appropriate primer mixes in each tube
3. Add 5 µl of the Reference DNA/PCR buffer from the Reference template into the SSP reaction tray. A multichannel pipette may be used for large panels.
4. Prepare a mix of water/Taq polymerase/ 60% sucrose or glycerol according to the following formula:

   n = number of tubes in template + 3

ddwater	n x 1.7 µl
60% sucrose or glycerol	n x 1.3 µl
Taq polymerase	n x 0.05

   Mix and add 3 µl to each tube of reaction tray.

   Total volume = 13 µl. Run the PCR program as usual for the SSP test.

   NOTE: The volumes indicated above may need to be modified slightly if using a commercial kit that requires different volumes. It is important to add about 70-100 ng of reference DNA per tube and then follow the same procedure that that is recommended for the kit being used.
5. Negative Control
   The SSP panel is tested with two or more cells that do not react with the same mixes to show that the primers are specific. Only control bands should be present in the negative tubes. If a specificity problem is suspected, or if a primer mix has been known to be troublesome in the past, the primer mix should be tested with a known Reference DNA that is very close to the specificity of the primer mix to ensure specificity (i.e. run allele 0402 against 0403 primer mix to show specificity with a closely related allele).
6. Complete Typing of Reference DNA
   In addition, a single Reference DNA may be run with a full set of primers (complete typing). The value of a full typing is that one can more effectively evaluate the presence of nonspecific bands and/or cross-reactive products. In addition, the presence of all the expected bands for a known type can be assured. This is especially valuable when designing a new panel or primer mix or when a problem arises which requires that the specificity of a primer mix be verified.
7. When performing quality control on a reagent, all other reagents used in the procedure must have been previously tested and found satisfactory.
8. It is also a good idea to repeat the QC in parallel with the next lot to document the stability of the reagents during storage and as a comparison with the new lot. Once the storage conditions have been validated, the end-of run parallel testing does not have to be continued unless the storage conditions are changed.

B. Monitoring of Primer Mix Reactivity

1. All aberrant results observed during the use of a lot of primer mixes should be recorded.
2. Continuous review of these reactions is necessary to determine the cause for the discrepancies (ex. cross-hybridization with similar sequence on another allele). Knowledge of aberrant reactions is vital when interpreting results.
3. The identification of new reaction patterns should be documented.

Probe QC for DNA Typing

A. SSOP Probe Labeling QC

1. After labeling, each probe is tested with reference DNA to ensure sensitivity and specificity of the reaction.
2. The results are recorded on the probe QC worksheet.
3. It is recommended that a panel of reference DNA be included with each SSOP run.

B. Reverse SSOP

1. For reverse SSOP developed in-house, new lots must be validated with sufficient reference DNA to ensure proper reactivity with each probe.

2. For commercial DNA typing kits, a reference DNA should be run prior to use with patient samples. Additional reference DNA should be tested periodically to monitor performance of the probes. The reference DNA should be rotated so that in the course of the year, most of the probes have been tested.

C. SSOP and Reverse SSOP Primer QC

1. After PCR, the PCR product is run on gel electrophoresis to determine if amplified product of the appropriate size is obtained. No further testing is done (Dot blot or ELISA) if no product is observed.
2. If no product is observed, one must troubleshoot to determine if the problem lies in the DNA sample or with one of the components of the PCR mix.

Titration of FITC-anti-human IgG for Flow Crossmatching

A. Goat Anti-human IgG

1. Anti-IgG heavy chain or Fc-specific reagent coupled with FITC
2. $F(ab')_2$ fragments have lower background binding
3. Specificity important – should not react with other Ig classes; affinity purified – pre-absorb with other Ig classes coupled to solid phase support

B. Titration of Anti-human IgG

1. Usually purchased in 1 mg vial. Reconstitute with 0.75 ml of H_2O and 0.75 ml of glycerol. Store in 10 µl aliquots in freezer
2. Concentration of reconstituted anti-IgG = 1 mg/1.5 ml = 0.67 mg/ml = 0.67 µg/µl
3. For titer, one should cover a range from 0.2 µg to 1 µg per test.
   The optimal amount is also dependent on the final volume of test.
   For example, one may wish to add 20 µl of the working dilution of the FITC-anti IgG to each test. If 20 µl contains 1 µg, then 1 µl would contain 0.05 µg.

   Dilution needed to make 0.05 µg/µl: 0.67 mg/ml ÷ 0.05 = 14.5

   Make 1:14.5 dilution of stock by adding 135 µl PBS to 10 µl stock = 0.05 µg/µl
4. The working dilution made above is then further diluted (ex. 1:2, 1:3, 1:4) and 20 µl of each dilution is used per tube in the crossmatch test. This should give a range from 0.25 to 1.0 µg per test when 20 µl of each working dilution is used.
5. Examine data and choose which working dilution gives optimal result. The dilution to use in future tests will be 14.5 x the secondary dilution used. For example, if the 1:3 dilution of the original 1:14.5 dilution gave the optimal results, then 3 x 14.5 or a working dilution of 1:43.5 should be made (10 µl aliquot + 425 µl PBS)
6. Document results of titer in Reagent QC record. Compare performance of new lot with old lot.

Equipment Maintenance

1. Written protocols for Preventive maintenance
2. Written schedule for maintenance checks – incorporate required frequency of maintenance checks
3. Documentation of maintenance checks – results recorded and stored in Maintenance Manual
4. Tolerance limits set for each maintenance check. The tolerance limits should appear on the worksheet on which the results are recorded.
5. Corrective actions and follow-up when results are outside tolerance limits.
   a. Written procedure for troubleshooting problem
   b. Written procedure for repairing instrument (if applicable)
   c. Back-up procedure or instrument
   d. Notification of proper persons with details of malfunction
   e. Back-up plan in case of power failure

References

1. ASHI Laboratory Manual, 3rd Edition, 1994. Section VI.6 Quality Control.
2. Standards, ASHI, 1996.
3. CAP Inspection Checklist, 1996.
4. ASHI Accreditation Standards Guidelines and Checklist, March 15, 1995.
5. DCI Laboratory Procedure Manual, Nashville, TN 1998

Example 1

REAGENT PRODUCT INFORMATION

Name	Manufacturer	Health Hazard	Preparation	Storage Req.	
				Temp	Shelf Life
Sodium Citrate, $Na_3C_6H_5O_7.2H_2O$	Sigma	skin irritant	stock chemical	22° C	indef.
PBS / 0.6% Citrate	Fisher	skin irritant	6 g NaCitrate dissolved in 1 liter PBS	0-8° C	6 mo.
EDTA, $CH_{10}H_{14}N_2O_8Na_2.2H_2O$	Fisher	skin, eye irritant	stock chemical	22° C	indef.
PBS/8% EDTA	Fisher	skin, eye irritant	40 g Disodium EDTA dissolved in 500 ml PBS. pH to 7.4 with NaOH.	2-8° C	6 mo.
Sodium Hydroxide, NaOH	Fisher	caustic	30 g NaOH dissolved in 100 ml deionized water	22° C	1 year

Example 2

REAGENT / MEDIA LOG and QUALITY CONTROL

_____ PBS _____ PBS/0.6% Citrate
_____ McCoy's Media _____ McCoy's with AB serum
_____ Fluoroquench _____ LSM
_____ Dynal Beads I / II _____ Other _____

Manufacturer	
Lot Number	
Previous Lot Number	
Received / Prepared Date	
Expiration Date	
Date Placed into use	

Quality Control Checks:

1. **Lymphocyte Processing**
The percentage of cell viability of a cell preparation using the new reagent is a reflection of its performance.

Tolerance Limit: Viability should be > 90%
Results: % Cell Viability = _____ Pass / Fail

Tech: _____ Date: _____

2. **Cytotoxicity Assay**
Processing reagents or media utilized in the lymphocytotoxicity test must show a score of "1" for the Negative control (AB serum) and a score of "8" with the positive control (ALS). Results are recorded for six consecutive tests.

Results:

Negative Control						
Positive Control						

Tech: _____ Date: _____ Pass / Fail

3. **pH** = _____ (PBS/0.6% Citrate = pH 7.0 – 7.2) Pass / Fail
(McCoy's Media = pH 6.6 – 7.1)

Tech: _____ Date: _____

Reviewed by: _____ Date: _____

Example 3

MISCELLANEOUS REAGENT QC

Year:_____

Reagent	Lot (Date Made)	Date Tested	Sample Tested	Pass/Fail	Tech	Review

COMPLEMENT TITER

Manufacturer: _____ Date tested: _____

Lot # : _____ Exp. date:_____ Tech: _____

Antiserum spec. _____ ID:_____

Cell phenotype: _____

			Complement Dilutions					
			A	**B**	**C**	**D**	**E**	**F**
	Serum Dilution		**Neat**	**1:2**	**1:4**	**1:8**	**1:16**	**Normal Serum No C′**
Neat	1	C′ Control; Buffer instead of serum						
Neat	2	Neg Control						
Neat	3	Antiserum						
1:2	4	"						
1:4	5	"						
1:8	6	"						
1:16	7	"						
Neat	8	Pos Control						
Neat	9	B cell Control						
	10							
	11							
	12							

Results:
C′ titer = _____

Comparable to old lot? Yes / No Acceptable? Yes / No

Reviewed by: _____ Date: _____

ANTIGLOBULIN TITER

Manufacturer: _____ Date tested: _____

Lot # : _____ Exp. date:_____ Tech: _____

Antiserum spec. _____ ID:_____

Cell phenotype: _____

			AHG Dilutions					
			A	**B**	**C**	**D**	**E**	**F**
	Serum Dilution		**Neat**	**1:2**	**1:4**	**1:8**	**1:16**	**B cell Control**
Neat	1	Pos Control						
Neat	2	Neg Control						
	3	Antiserum, no AHG						
1:20	4	"						
1:40	5	"						
1:60	6	"						
1:80	7	"						
1:100	8	"						
1:120	9	"						
1:140	10	"						
1:160	11	"						
1:180	12	"						

Optimal AHG dilution = _____

Comparable to old lot? Yes / No Acceptable? Yes / No

Reviewed by: _____ Date: _____

Synthesis of Rare DRB1 Alleles for SSOP

Debra D. Hiraki, Shalini Krishnaswamy and Carl F. Grumet

Principle and Purpose

Validation of any HLA typing method should demonstrate that the test is capable of reacting properly with all alleles it claims to be able to identify. For example, if the assay is based on oligonucleotide probes (SSOP) recognizing short stretches (~20 bp) of DNA within a PCR product, the assay is best validated if the probe can be tested against all the sequence variations known to exist within the target sequence of the probe. Generally, differences outside of the probe site are not as important to probe reactivity as those within the probe site. Further, some probes may not hybridize as predicted, and therefore probe reactivity should always be verified under actual test conditions. Oligonucleotides are sometimes substituted as test targets in SSOP validations; however their reactivity may differ substantially from that of the whole amplicon, necessitating the use of real PCR amplicons. Since many alleles currently recognized are very rare, finding test DNAs to use for validation of all alleles may be difficult or impossible.

An alternative to finding the rare alleles is to simply synthesize them. Rare alleles are generally very close in sequence to some common alleles, differing in only one or two bases. End differences, i.e. those present within 30 base pairs of a primer site can be incorporated into the synthetic product simply by using a newly designed, extended primer in a second round of PCR amplification (We have used this technique to generate DRB1*1316, *1328 and *0423 amplicons). For middle differences, i.e. those more internal than 30 base pairs, the similarity to common alleles can be exploited by using the technique of overlap PCR. This technique utilizes two internal primers to introduce the rare allele's DNA sequence into it's closest common allele to yield a product similar (or identical) to that used in the assay. Figures 1 and 2 illustrate overlap PCR applied to the synthesis of a DRB1*1426 product. (We have also used this method to generate DRB1*0703 and *1506 amplicons.) Synthesis of rare DR alleles is thus feasible and offers the best available test material for complete validation of molecular typing methods.

Specimen

The initial amplicon for this procedure needs to be a single DRB1 allele closest in sequence to the desired rare allele (i.e. differing in only 1 or 2 closely positioned base pairs within the entire amplicon.) The starting genomic DNA chosen to produce the initial amplicon therefore must be of an HLA type that not only possesses the desired closely related allele, but also is either homozygous for DRB1 or possesses a second allele that will not amplify with the chosen primers. Furthermore, the primers should be chosen so that there will be no amplification of DRB3, 4 or 5 locus products. For example, when the rare DRB1*1426 was sought, the GH46-CRX37 primer pair could be used with any DR2, DR1401 heterozygote since that primer pair amplifies only DRB1 products, but not DR2, 7 and 9 alleles.

Figure 1. Genomic and primer sequences for the creation of DRB1*1426
This figure shows the relevant genomic sequence for DRB1*1426 and the closely related, more common allele DRB1*1401. The biotinylated sense and antisense primers were designed to introduce into a 1401 amplicon the new A in codon 24 of 1426 using overlap PCR.

RELEVANT GENOMIC SEQUENCES:

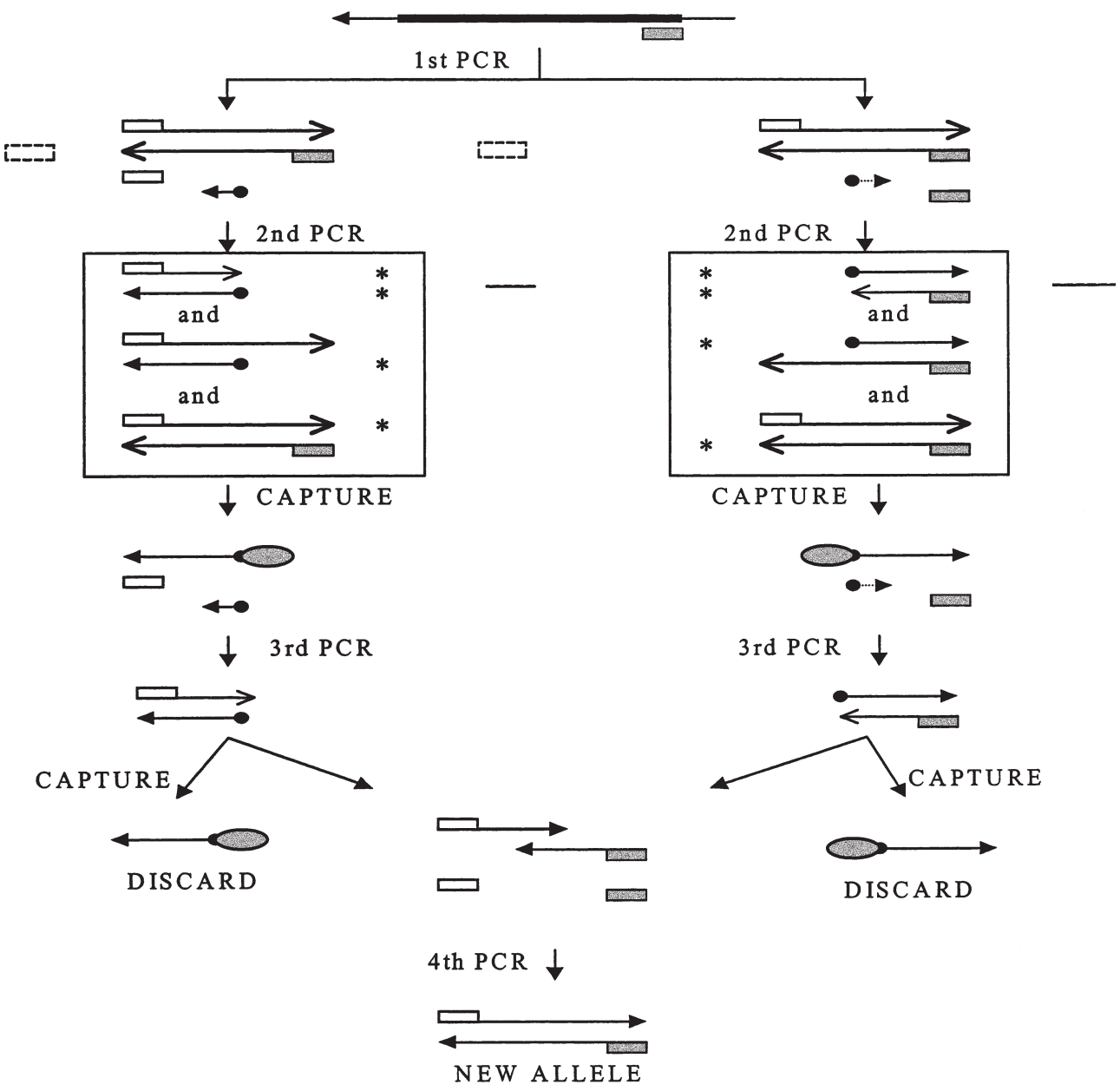
DRB1*1426: ...5′ TGG GAC GGA GCG GGT GC**A** GTT CCT GGA CAG ATA CT...

DRB1*1401: ...5′ TGG GAC GGA GCG GGT GC**T** GTT CCT GGA CAG ATA CT...

PRIMERS:

BIOTIN-DR1426 Sense	5′ Bio-GA GCG GGT GC**A** GTT CCT GGA C 3′
BIOTIN-DR1426 Antisense	5′ Bio-GA GCG GGT GC**T** GTT CCT GGA C 3′
CRX37	5′ GAA TTC CCG CGC CGC GCT 3′

Figure 2. The generation of a synthetic DRB1 allele:



Fuller length, non-mutated fragments persist and are generated in the early steps. These non-mutated fragments could subsequently increase the background of unmodified, original amplicon and interfere with the duplexing of the desired mutated half-strands. To eliminate the contamination, the mutated strands are isolated on streptavidin-coated magnetic beads. The duplex is denatured and all contaminating strands are removed. A third round of amplification yields pure mutated products. After another round of capture and denaturation, the biotinylated strands are discarded and the non-biotinylated strands are allowed to duplex to form the template for the new allele, amplified with the original primers. GH46; CRX37; SA streptavidin-coated magnetic beads; * newly generated strands in this round of PCR; biotinylated antisense DRB1*1426 primer; biotinylated sense DRB1*1426 primer.

Reagents and Supplies

Primers:

1. Redesigned primer for one end extended in the 3′ direction to include the new desired allelic bases
   OR
   Biotinylated 20-mer primers in both the sense and antisense directions, completely overlapping and designed with the desired new allelic base substitution(s) centered in both primers.
2. Streptavidin-coated Dynabeads M-280
3. Standard PCR reagents
   a. TEN (10mM Tris, pH 7.5; 1mM EDTA; 2M NaCl)
   b. 0.1 N NaOH
   c. TE (10mM Tris, pH 7.5; 1 mM EDTA)
   d. 0.8 N HCl
   e. Standard agarose gel

Instrumentation and Special Equipment

Magnet for Dynabead separation
Thermocycler
Agarose gel electrophoresis equipment

Quality Control

Prepare a substantial amount of product for future use and store aliquots at -70° C. Use as reference DNA with quality control of new probe mixtures.

Procedure

FOR ALLELES WITH NEW POLYMORPHIC POSITIONS WITHIN 30bp OF A PRIMER:

1. Redesign the closest primer to extend up to (and, if necessary, past) the sites of the desired introductions, up to 45bp in length. If the final primer is too long, the primer may be then shortened on the 5′ end to make a usable primer. The final product will then be just a few bases shorter than the regular test amplicon.
2. Amplify with your regular primer pair (as discussed under Specimen.)
3. Dilute the product 10^{-5} to 10^{-7} and reamplify with the newly designed primer and the original primer going in the other direction. Verify clean amplification on an agarose gel.

FOR ALLELES WITH NEW POLYMORPHIC POSITIONS MORE THAN 30bp AWAY FROM A PRIMER: (The following steps are diagrammed in Figure 2.)

1. Amplify the chosen genomic DNA with your regular primer pair (as discussed under Specimen.)
2. Dilute the original product 10^{-4} to 10^{-6} and reamplify to give 2 fragments:
   a. Original left hand primer (sense) with the new biotinylated antisense primer to give a left hand product.
   b. Original right hand primer (antisense) with the new biotinylated sense primer to give a right hand product.
   c. These two new products overlap and are complementary on the 3′ terminus of their mutated strands. Verify clean, single band amplification for each on an agarose gel.
3. Isolate biotinylated strands from contaminating whole, non-mutated strands:
   a. Prepare 2 aliquots of 20 µl avidin-coated Dynabeads per manufacturer's instructions.
   b. Resuspend each aliquot of beads in 40 µl TEN and mix one with 40 µl left hand product and the other with 40 µl right hand product.
   c. Bind 15 min, room temperature with rotation or occasional shaking. Wash with 40 µl TEN.
   d. Denature the non-biotinylated strand with 10 µl 0.1 N NaOH for 10 min, room temperature.
   e. Remove the NaOH containing the nonbiotinylated strand.
   f. Wash the beads with 50 µl 0.1N NaOH, followed sequentially by 50 µl TEN, 50 µl TE and final resuspension in 40 µl DDW
4. Amplify only mutated templates: Dilute beaded biotinylated products 10^{-2}. Repeat last pair of amplifications. Verify clean, single-band amplification on an agarose gel.
5. Stitch together the proper fragments: Since now only mutated fragments are present and since the two fragments are complementary, a new template DNA can be generated by allowing the fragments to anneal at their mutated ends, i.e. duplexing the non-biotinylated strands from each reaction.
   a. Prepare 40 µl avidin-Dynabeads as above with resuspension in 80 µl TEN.
   b. Mix **both** products (40 µl each) and beads together and bind 15 min, room temperature.
   c. Wash the beads with 100 µl TEN.
   d. Denature with 20 µl 0.1 N NaOH. **Remove and save the NaOH supernatant with the nonbiotinylated strands to a new tube.**
   e. Neutralize immediately with 3 µl 0.8 N HCl.
   f. Dilute with an additional 50 µl water or 10 mM Tris, pH 7.5.

6. This mixture does not store long. Amplify immediately at 10^{-1} to 10^{-4} dilution of above mixture with original primers (e.g., CRX37 – GH46) to identify the best dilution for amplification. Verify clean amplification on an agarose gel. Amplify a large quantity of product for use and storage.

Results

The new product should now contain the desired allele sequence. Verify by sequencing. Use this new product in the validation of any assay required. Because this product will be very pure, be sure to use a suitable dilution in your validation assays.

Procedure Notes

1. If the products at any stage are not single bands for some reason, it may be necessary to run the product on an agarose gel, cut out the desired band and purify it on a spin column before proceeding with the Dynabeads and subsequent amplification.
2. Although this procedure was used to synthesize oligonucleotides that can be used for an SSOP method, it may possible to use this product with SSP assays as well. However, in order to prevent cross-hybridization and false positive results, one must optimize the dilution of the synthesized product. In addition, the synthesized oligo should be mixed with DNA from a cell containing a similar allele in a proportion that would represent its normal frequency in a DNA extract.

Limitations of Procedure

1. Failure to find a starting DNA of a type which will allow the single, unique amplification of one desired DRB1 allele or the use of primers which amplify anything in addition to the one DRB1 allele will result in a mixture of products and inaccurate validation.
2. Titration of the synthesized product is necessary to determine the optimal dilution for best sensitivity and specificity.

References

1. Horton RM, Hunt HD, Ho SN, Pullen JK and Pease LR, Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. Gene 77: 61-68, 1989.
2. Behar, E., Lin, X., Grumet, F.C., Mignot, E. A new DRB1*1202 allele (DRB1*12022) found in association with DQA1*0102 and DQB1*0602 in two Black narcoleptic subjects. Immunogenetics 41:52, 1995.

Quality Control for DNA Contamination

Jeffrey M. McCormack

Principle

The polymerase chain reaction is a very powerful tool that can be used to amplify segments of DNA a million-fold or more. One of the dangers of using this technique is contamination of the laboratory with amplicons which can be re-amplified in subsequent PCR runs. An important part of quality assurance in laboratories performing PCR is to monitor for DNA contamination. DNA contamination, either genomic or amplicon, could conceivably yield false positive results, and as a consequence, erroneous reporting. Therefore, strict criteria have been established for molecular typing laboratories to perform routine tests aimed at identifying DNA contamination.[3]

Acceptable means for controlling DNA contamination include the use of ultraviolet (UV) irradiation,[7,8] uracil-DNA glycosylase,[9-11] hydroxylamine hydrochloride[12] and exonuclease III.[13] While these methods are in most cases adequate, it is still important to have a reliable method to monitor the effectiveness of de-contamination efforts and to identify potential problems with contaminating DNA or amplicons. Laboratories performing molecular histocompatibility typing are required to monitor DNA contamination by regular wipe tests, testing negative controls (no DNA), open tubes, etc.[3] The purpose of the wipe test is to survey laboratory surfaces and equipment for DNA contaminants and then take appropriate steps to decontaminate areas which test positive. Similarly, the use of open tube controls and negative controls provide a means to monitor for aerosolized DNA and contaminated reagents, respectively.

Appropriate objectives to effectively monitor contamination include 1) the design of an oligonucleotide primer set specific for nonpolymorphic regions of class I and/or II for use as a control primer set; 2) establish and validate a PCR-based wipe test procedure and 3) verify the use of the primer set for detecting PCR products generated by the method being used.

To monitor for Class II amplicons, a primer set, RBQBf/RBQBr was developed which is specific for nonpolymorphic regions of the DR-, DQ- and DP- consensus sequences. The expected PCR products are 81 bp (DR- and DP-) and 79 bp (DQ-). RBQBf/RBQBr detects genomic DNA from reference cell lines LWAGS and BM21 (50-100 picograms) as well as DR-, DP- and DQ- amplicon (1 copy). Additionally, RBQBf/RBQBr detects SSP-PCR products from clinical DR- and DQ-class II typings.

Validation studies employing controlled DNA contamination of laboratory surfaces revealed that increasing amounts of wipe test sample (5-20%) were inhibitory to the wipe test PCR, whereas lower amounts (1-2%) or, alternatively, a diluted wipe test sample, increased the sensitivity of the test and optimized the results. It was also observed that inhibitory factors introduced into the PCR during the wipe test process may yield false negative results. The Wipe Test must be designed to have optimal sensitivity and the validity of negative results must be confirmed by testing for inhibitory factors. This is routinely done by spiking a second PCR test with a known amount of DNA amplicons.

Materials and Reagents

Wipe Test Primers

RBQBf	GCT TCG ACA GCG ACG TG
RBQBr	CCT TCT GGC TGT TCC AGT ACT C

Wipe test PCR mix
1x PCR buffer
500 µM deoxynucleotide triphosphates (dNTP's)
2.5 mM $MgCl_2$, 1x PCR buffer
0.5 µM of each oligonucleotide primer
Taq polymerase
Agarose gel
4% agarose gel made with 3:1 Nusieve Agarose (FMC Bioproducts, Rockland ME) or 2% agarose
Positive Control –
a pre-amplified PCR product is serially diluted from 1:1000 to 1:1,000,000 and tested with the wipe test PCR mix. The product should be detected up to at least 1:10,000. Choose the highest dilution that gives a strong positive band as the working dilution to use for the positive control in the Wipe test.
Filter paper or a Puritan cotton-tipped applicator
(3 in.; Hardwood Products Company, Guilford, ME) for wiping test areas
Forceps
Purified, nitrocellulose-filtered water
Isopropanol

PCR Cycling Conditions
95° C, 30 seconds
60° C, 15 seconds
72° C, 15 seconds for 30 cycles

Procedure

Wipe tests should be taken from the DNA isolation area, the PCR set-up area, the clean room bench area, the floor of the clean room, the reagent preparation area, the thermal cyclers, and the electrophoresis area.

Each wipe test sample is amplified with the designated "wipe test primers" that are capable of detecting all PCR products as well as genomic DNA contamination.

The internal control primers are also included in the wipe test primer mix. A duplicate PCR test is set up which is spiked with DNA or a dilution of PCR product. This is run to ensure that there is not extraneous matter in the wipe test sample that is interfering with or inhibiting the Taq polymerase. Score "+" or "-" for presence or absence of a PCR product on the gel.

A. Wiping Procedure

1. Decontaminate forceps isopropanol and rinse in ultrapure water or use sterile disposable forceps.
2. Wet 1.5 cm diameter disk of filter paper in ultrapure water using the forceps.
3. Wipe filter paper or swab over a 10 cm square area.
4. Place filter paper or swab in a 1.5 ml microfuge tube with 120 µl ultrapure water and vortex.
5. Incubate at 56° C for 1 hour. Centrifuge at 7000 rpm for 30 seconds. Store in refrigerator until tested.

B. PCR for Wipe Test

1. Aliquot 8 µl Wipe Test PCR mix into 16 PCR tubes. Also add the Wipe test PCR mix to a tube that has been opened on the work area for at least one day (Open tube control).
   NOTE: It is suggested that a batch of Wipe test PCR mix be made and pre-aliquotted into strips of PCR tubes. These can be stored frozen until needed. The mix will need to be added to the Open Tube control on the day of testing.
2. Arrange the tubes for one Positive, one Neg (No DNA), one test sample for each area wiped, one spiked sample for each area wiped, and one Open tube Negative.
3. Add 2 µl of supernatant from each wipe test sample to the appropriate duplicate tubes.
4. In a separate tube, mix 40 µl sucrose or glycerol with 1 µl Taq polymerase. Add 2 µl to each of the tubes.
5. Add 2 µl of known positive sample to the Positive control tube and to one of the duplicate wipe test samples.
6. Amplify the wipe test samples and controls using the lab's standard amplification protocol.
7. PCR products can be electrophoresis on a 4% agarose gel made with 3:1 Nusieve Agarose or a 2% agarose and subsequently visualized and documented by ethidium-bromide staining, UV transillumination and photography.

Results

The results are recorded on a worksheet.

Area Wiped	PCR Result	Spiked PCR Result
DNA Isolation Hood		
PCR Set-up Hood		
Reagent Preparation Area		
DNA Clean Room Floor		
Thermal Cycler Area		
Fume Hood / Gel Preparation Area		
Post-amplification Bench Area		

Positive Control _____ Open Tube Negative Control_____ Pass / Fail

Contaminated Areas

Contaminated areas should be cleaned thoroughly with 1M HCl or 10% bleach. Wipe tests should be repeated and should be negative (with exception of possibly the post-amplification areas) before work continues.

Contaminated Area	Date Cleaned	Retest Results

Interpretation

1. There should be a PCR product present in the Positive control tube. No product should be present in the Negative control.
2. There should be a PCR product in the "spiked" tubes for each of the wipe test areas. The absence of a PCR product in these tube suggests that the reaction may have been inhibited by materials present in the wipe test sample. If the spiked sample fails to show a product, the corresponding "unspiked" wipe test cannot be interpreted.
3. The presence of a PCR product in the unspiked wipe tests indicates contamination with genomic DNA or amplicons. De-contamination procedures should in instituted immediately and the wipe test repeated to verify that the contaminants have been successfully removed.

Quality Control

1. A Positive control is included with each run. The positive control can be genomic DNA (25 ng/µl) or a dilution of PCR product to test the ability of the primers to detect contamination.
2. A Negative Control and/or Open tube negative control is included with each run. The negative control contains no known source of DNA and is used to identify contamination in reagents used in the test or from aerosols (open tube control).
3. Spiked controls are set up with each of the test samples. A duplicate of the test sample is spiked with a known amount of positive control. Failure of the spiked sample to amplify suggests that there may have been something picked up from the wipe test that is inhibiting the reaction. For example, bleach residue has been known to inhibit the polymerase reaction and thus invalidate the test.

Validation Procedures

Introduction

When the RBQBf and RBQBr primers were first designed, it was necessary to validate their ability to detect low amounts of DNA contamination, both genomic and amplicon. The following describes the procedures that were undertaken to validate this test. It is not necessary for each laboratory to repeat this validation if using the same wipe test primer set. However, if additional primers are needed (for example, to detect Class I amplicons), a similar approach may be taken.

RBQBf/RBQBr Primer Design

An optimal set of primers for use in monitoring DNA contamination would identify genomic DNA as well as amplified PCR products generated from the polymorphic region of exon 2 of the HLA class II B genes. Examination of the polymorphic and nonpolymorphic regions of exon 2 revealed areas which would serve as likely primer annealing sites and meet these criteria. The polymorphic regions include: DR- (amino acid positions 9-13, 25-38 and 67-74), DQ- (amino acid positions 26-37, 52-57, 70-74) and DP- (amino acid positions 8-11, 33-36, 55-57, 65-69, 72-76 and 84-87). The nonpolymorphic regions nested between areas of polymorphism were selected as potential targets for wipe test oligonucleotide class II primer annealing sites. Using PRIMER ((Version 0.5 by Stephen E. Lincoln, Mark J. Daly and Eric S. Lander, MIT Center for Genome Research and Whitehead Institute for Biomedical Research), a software package for designing oligonucleotide primers, RBQBf/RBQBr primer set was selected. The forward primer (RBQBf) anneals at nucleotides encoding amino acids 39-44 for DR- and DQ-, and 37-42 for DP-. The reverse primer (RBQBr) anneals at nucleotides encoding amino acids 59-66 for DR- and DQ-, and 57-64 for DP-.

WQLKF/G86r primer set are modified oligonucleotides previously reported[9] and amplify DRB1*0101/0103 alleles yielding a 257 bp product.

DPAMP-A/DPAMP-B primer set generates a 327 bp product and are generic primers which amplify all DPB1 alleles.

QB1D/GILQRR primer set are modified oligonucleotides previously reported[10] and amplify DQB1*05/06 resulting in a 268 bp product.

All PCR primers were synthesized using Applied Biosystems 380B and 381A DNA Synthesizers.

Control DNA

Homozygous typing cells used for characterizing RBQBf/RBQBr primers were:

KOSE (A2, B35, Cw12, DRB1*1302/1401, DQB1*05031/0604),
WT100BIS (A11, B35, Cw4, DRB1*0101, DQB1*0501), and
BM21 (A1, B41, Cw17, DRB1*1101, DQB1*0301).

Genomic DNA from patients samples were isolated using Genomix (Washington Biotechnology, Bethesda MD) per the manufacturers instructions. Genomic DNA from homozygous typing cells was isolated by phenol/chloroform extraction.

DR-, DQ- and DP- Amplification and Purification

1. DR-, DQ- and DP- amplicons were generated using various oligonucleotide primers previously reported.[10]
2. The forward primer (RBQBf) anneals at nucleotides encoding amino acids 39-44 for DR- and DQ-, and 37-42 for DP-. The reverse primer (RBQBr) anneals at nucleotides encoding amino acids 59-66 for DR- and DQ-, and 57-64 for DP-. RBQBf/RBQBr generates 81 bp PCR products from DR- and DQ-, and DP-.
3. PCR conditions were identical to those described for RBQBf/RBQBr with the exception of the cycling conditions. PCR cycling conditions were:
   1) DR-, 95° C, 30 seconds; 60° C, 15 seconds; 72° C, 15 seconds for 30 cycles
   2) DP- 95° C, 30 seconds; 70° C, 30 seconds for 30 cycles
   3) DQ- 95° C, 20 seconds; 55° C, 50 seconds; 73° C, 20 seconds for 34 cycles.
4. The purification of PCR products was accomplished by excising the desired bands from the agarose gel following electrophoresis and then using Wizard PCR Preps DNA Purification System (Promega Corp., Madison, WI).

SSP-PCR Typing

1. SSP-PCR typing was performed using the UCLA PCR-Amplification Mixtures (UCLA Tissue Typing Laboratory, Los Angeles, CA). as described by the manufacturer.
2. The PCR was performed using a Perkin-Elmer 9600 thermal cycler for 32 cycles using the following cycling parameters: 95° C, 15 seconds; 58° C, 15 seconds; 73° C, 10 seconds.
3. PCR products were analyzed by electrophoresis and photographed as described above. For analysis of PCR products, generated bands were excised from the agarose gel, melted and used as template DNA for RBQBf/RBQBr amplification.

Validation Test

1. In order to validate the established wipe test procedure, known amounts of genomic and amplified DNA were used to contaminate laboratory surfaces.
2. Ten-fold serial dilutions (250 µl) of genomic DNA or amplicon were applied to previously decontaminated laboratory bench surfaces and allowed to dry overnight.
3. The samples were applied to defined benchtop areas to ensure that DNA would be acquired during the validation procedure. The samples were recovered and processed as described above in the wipe test procedure.
4. Experiments assessing the presence of inhibitory factors in wipe test samples were accomplished by mixing varying amounts of a wipe test sample with known amounts of amplified DNA.
5. In each case the PCR product was quantitated using NIH Image, a public domain program designed for digital image processing and analysis.
6. The integrated intensity of each band was determined and the percent inhibition was calculated using as 0% inhibition a PCR reaction without wipe test sample.

Results

The expected PCR product generated from DR-, DP- and DQ- class II genes is 81 bp. The PCR products generated using primer sets WQLKF/G86r, DPAMP-A/DPAMP-B and QB1D/GILQRR will result in products which include the non-polymorphic regions recognized by RBQBf/RBQBr primer set, therefore making these PCR products useful tool for evaluating the effectiveness of RBQBf/RBQBr in detecting DR-, DQ-, DP- and amplicons.

A. Detection of Genomic and Amplified DNA Using RBQBf/RBQBr Primer Set

The sensitivity of the primer set RBQBf/RBQBr was first determined by testing serial dilutions of target genomic DNA from reference cell lines LWAGS and BM21. Using two-fold serial dilutions of genomic DNA, it was determined that the primer set was capable of detecting between 50-100 picograms of genomic DNA. Likewise, purified DRB1*0101 amplicon was quantitated and used as target DNA and RBQBf/RBQBr was able to detect a single copy of purified DRB1*0101 PCR product. Similar results were obtained using purified DP- and DQ- amplicon, thus demonstrating that the primer set RBQBf/RBQBr is capable of detecting low levels of both genomic (50-100 picograms) and amplified DNA (single copy).

B. RBQBf/RBQBr Detection of DR-, DP- and DQ- Amplicon

Using target DNA from reference cell lines WT100BIS and KOSE and a patient sample, PCR products were generated for DRB1*0101 DQB1*05031/0604, DP- 1 respectively, using primer sets previously described. Amplicon were purified as described in Materials and Methods and used as target DNA to assess whether RBQBf/RBQBr primer set could detect amplicon generated from the class II genes DR-, DQ-, and DP-. PCR products generated using RBQBf/RBQBr to detect amplicon clearly showed that RBQBf/RBQBr satisfactorily detects all three amplicon. These data demonstrate that RBQBf/RBQBr will serve as a mechanism for detecting PCR products generated from all class II genes.

C. Inhibition of Amplicon Detection with Increasing Wipe Test Sample Volume

In order to verify the effectiveness of the RBQBf/RBQBr primer set, a validation process was established which consisted of controlled contamination of laboratory surfaces and subsequent detection of the contamination using the wipe test procedure. However, a significant observation made in the initial phase of the validation protocol was that when using published procedures calling for 20% of the PCR test to be wipe test sample,[3] false negative results were consistently observed from areas known to be contaminated. One approach to explaining the observed false negative

results was to determine whether inhibitory factors from the wipe test samples were being introduced into the PCR-based test. To test this hypothesis, varying amounts of a routine wipe test sample (2-20% final PCR volume) was added to known amounts of amplicon to determine if the test samples would inhibit the PCR. When using 20,000 copies of DRB1*0101 amplicon as target DNA, and 20%, 15% or 10% of the PCR volume consisting of wipe test sample, 100% inhibition of the PCR was observed. Inhibition of 90% was observed using 5% sample and 48% inhibition when 2% of the final volume was the wipe test sample. These data clearly demonstrate that significant amplicon contamination (20,000 copies) may yield false negative results when wipe test samples are added at increasing amounts (5-20%). Moreover, it is possible that lower levels of DNA contamination might go undetected using wipe test samples equal to or less than 1-2% of the PCR. For example, a single amplicon contaminating a surface might go undetected due to inhibitory factors with the addition of less than 1% of wipe test sample.

D. Detection of SSP-PCR Typing Amplicon

The primer set RBQBf/RBQBr was able to detect low levels of both genomic and amplified DNA. However, the definitive test to assess the value of RBQBf/RBQBr as tools to monitor DNA contamination in the molecular typing lab was to determine the effectiveness in detecting PCR products generated in routine laboratory typings. To accomplish this, random SSP-PCR products were sampled from an SSP-PCR typing methodology, the UCLA PCR-Amplification Mixtures from the UCLA Tissue Typing Laboratory, Los Angeles, CA. The results of sampling PCR products generated from a clinical typing and then using the amplified PCR product as target DNA for RBQBf/RBQBr. Samples which were selected indicated that the PCR results when the samples were used as targets for RBQBf/RBQBr amplification. Clearly all PCR products generated from the typing served as a suitable template for RBQBf/RBQBr amplification. Taken together these results showed that RBQBf/RBQBr is an efficient primer set for detecting amplicon generated from SSP-PCR histocompatibility typing.

Discussion

The level of polymorphism of the human major histocompatibility complex (HLA) has historically been a major obstacle to generating thorough histocompatibility testing. Recently however, PCR-based approaches have exploited the genetic intricacy of the HLA complex in developing molecular typing methods which produce, in many cases, definitive results. While the results are indeed favorable, the use of PCR methods introduces a new set of QC issues relating to the increased sensitivity inherent to the PCR. It is imperative that laboratories adhere to strict guidelines regarding protective clothing, laboratory design and workflow to minimize potential DNA contamination. Moreover, laboratories are required to monitor DNA contamination by weekly wipe tests, utilization of open tube controls during DNA isolation and testing negative controls (no DNA) samples. Compliance with these regulations demands close scrutiny of the design, validation and implementation of QC procedures used in monitoring DNA.

References

1. Hurley, C, Yang SY: Quality assurance and quality control for amplification-based typing. ASHI Laboratory Manual, 1995, V1.13.1.
2. Ou, CY, Moore, JL, Schochetman G: Use of UV irradiation to reduce false positivity in the polymerase chain reaction. Biotechniques 10:442, 1991.
3. Pang J, Modlin J, Yolken R: Use of modified nucleotides and uracil-DNA glycosylase (UNG) for the control of contamination in the PCR-based amplification of RNA. Mol Cell Probes 6:251, 1992.
4. Thornton CG, Hartley JL, Rashtchian A: Utilizing uracil DNA glycosylase to control carryover contamination in PCR: characterization of residual UDG activity following thermal cycling. Biotechniques 13:180, 1992.
5. Longo MC, Berneinger MS, Hartley JL: Use of uracil DNA glycosylase to control carry-over contamination in the polymerase chain reaction. Gene 93:125, 1990.
6. Aslanzadeh J: Application of hydroxylamine hydrochloride for post-PCR sterilization. Mol Cell Probes 7:145, 1993.
7. Zhu YS, Isaacs ST, Cimino G, Hearst JE: The use of exonuclease III for polymerase chain reaction sterilization. Nucleic Acids Res 19:2511, 1993.
8. Sarkar G, Sommer SS: Parameters affecting susceptibility of PCR contamination to UV inactivation. Biotechniques 10:590, 1991.
9. Olerup O, Zetterquist H: HLA-DR typing by PCR amplification with sequence-specific primers (SSP-PCR) in 2 hours: an alternative to serological DR typing in clinical practice including donor-recipient matching in cadaveric transplantation. Tissue Antigens 39:2257, 1992.
10. McCormack, JM, Sherman M, Mauer DH. Quality control for DNA contamination in laboratories using PCR-based class II HLA typing methods. Human Immunology 54 (1):82, 1997.

The Joint Commission on Accreditation of Healthcare Organizations

Anne Belanger

The Joint Commission evaluates and accredits nearly 20,000 health care organizations and programs in the United States. An independent, not-for-profit, Self-supporting organization, the Joint Commission is the nation's predominant standards setting and accrediting body in health care. Since 1951, the Joint Commission has developed state-of-the-art, professionally based standards and -valuated the compliance of health care organizations against these benchmarks. Joint Commission evaluation and accreditation services are provided for a wide-variety of health care organizations including hospitals, home care organizations, nursing homes, and many types of clinical laboratories.

The Joint Commission's corporate members are the -American College of Physician American Society of Internal Medicine, the American College of Surgeons, the American Dental Association, the American Hospital Association, and the American Medical Association. Governance consists of a 28-member Board of Commissioners including nurses, physicians, consumers, administrators, providers, employers, labor representatives, health plan leaders, quality experts, ethicists, health insurance administrators and educators. The board brings to the Joint Commission countless years of diverse experience in health care, business and public policy.

The Joint Commission accredits approximately 2,700 organizations that provide laboratory services., including independent laboratories and laboratories in other types of accredited health care organizations. Laboratories eligible for accreditation include:

- Laboratories in hospitals, clinics, long term care Facilities, home care organizations, behavioral health organizations, research labs, ambulatory sites and physician offices;
- Independent laboratories performing specialty testing of all types as well as routine testing-Blood transfusion and donor centers;
- Governmental laboratories, such as Indian Health Service, Veterans Administration and military outpatient laboratories.

The Joint Commission uses performance-focused standards that emphasize the results a laboratory should achieve, rather than specific methods of compliance. The standards manual contains many examples of how compliance might be achieved in various types of laboratory settings for each standard. Laboratories may follow examples as written, modify the examples to suit their own situation, or develop their own path to compliance. As long as the laboratory meets the intent of the standard, compliance is assured.

In 1995, the Joint Commission launched a cooperative accreditation initiative to reduce redundancy and overlap in the accreditation of health care organizations. The initiative focused on improving the efficiency, and reducing the cost of quality oversight activities by enhancing the communication and coordination among various public and private sector organizations that have responsibility for these activities. This initiative, cemented by written agreements, permits the Joint Commission to substantially rely on the process, findings, and decisions of other accrediting bodies in circumstances where the Joint Commission would otherwise conduct potentially duplicative surveys of organizations seeking accreditation.

Under these cooperative agreements, the Joint Commission will accept the accreditation decision of the other accrediting body or government agency for specific components of health care organizations undergoing Joint Commission review. For those Joint Commission standard areas not covered by the other accrediting body, the Joint Commission may conduct a limited survey.

Organizations with cooperative agreements have passed an extensive review of their standards and standards development process; survey process; selection, training and monitoring of surveyors; and accreditation decision process. They have also agreed to maintain an approach to public disclosure, comparable to the Joint Commission's approach. Beside the American Society for Histocompatibility, and Immunogenetics, the Joint Commission has also finalized cooperative accreditation agreements with seven other professional organizations with accreditation including American Association for Ambulatory Health Care (AAAHC), American College of Radiology Radiation Oncology Program, CARF, The Rehabilitation Accreditation Commission (Medical Rehabilitation Program), and the College of American Pathologists. The cooperative agreements with ASHI, CAP, CARF Medical Rehabilitation, COLA and CHAP apply to all accreditation programs. The cooperative agreements with AAAHC, ACR Radiation Oncology and CoC apply only to the Network Accreditation Program and will be reevaluated at a later date for applicability to other accreditation programs.

In addition, the Joint Commission has interimagreements with six other organizations which apply only to the Network accreditation Program. These interim agreements are currently being evaluated for potential future cooperative agreements.

For more information about the Joint Commission and all its accreditation programs, educational products and services, consumers and the health care community can access the web site at www.jcaho.org.

ASHI – The HCFA Perspective

Sandra Pearson and Esther-Marie Carmichael

What is DHHS?

The **DEPARTMENT OF HEALTH AND HUMAN SERVICES (DHHS)** is the government's principal agency for protecting the health of all Americans and providing essential services, especially for those who are least able to help themselves. The DHHS includes more than 300 programs, covering a wide spectrum of activities, such as, medical and social science research; infectious disease prevention (immunizations); assuring food and drug safety; Medicare and Medicaid health insurance programs; financial assistance for low-income families; child support enforcement; improving maternal and infant health, head start, preventing child abuse and domestic violence, substance abuse treatment and prevention, services for older Americans, comprehensive health services delivery for American Indians and Alaska Natives. The Office of the Secretary provides leadership.

Divisions under DHHS include:

National Institutes of Health
Centers for Disease Control & Prevention
Indian Health Service
Substance Abuse & Mental Health Services Administration
Health Care Financing Administration
Administration on Aging
Food and Drug Administration
Agency for Toxic Substances and Disease Registry
Health Resources & Services Administration
Agency for Health Care Policy and Research
Administration for Children and Families

What is HCFA?

The **HEALTH CARE FINANCING ADMINISTRATION (HCFA)** is the federal agency that administers the Medicare, Medicaid, and Child Health Insurance Programs. HCFA helps pay the medical bills for more than 75 million beneficiaries. HCFA also regulates all laboratory testing (except for research). Approximately 158,000 laboratory entities fall within HCFA's regulatory responsibility. HCFA's responsibilities include:

- assurance that the Medicaid, Medicare, and Children's Health Insurance programs are properly run by its contractors and state agencies;
- establishes policies for paying health care providers;
- conducts research on the effectiveness of various methods of health care management, treatment, and financing;
- assess the quality of health care facilities and services and taking enforcement actions as appropriate;
- areas of special focus:
  fighting fraud and abuse; and
  improving the quality of health care provided to the beneficiaries by:
  – developing and enforcing standards through surveillance;
  – measuring and improving outcomes of care;
  – educating health care providers about quality improvement opportunities; and
  – public education to encourage good health care choices.

HCFA's structure includes their headquarters located in Baltimore, Maryland, with 10 Regional Offices nationwide overseeing the HCFA programs. The headquarters staff are responsible for national program direction and national reporting. The Regional Office staff provides HCFA with the local presence necessary for quality customer protection and service and program oversight. The Regional Office locations are available on the Internet at www.hcfa.gov/medicaid/clia/cliahome.htm.

CLIA Authority

CLIA is the Clinical Laboratory Improvement Amendments of 1988. The responsibility for carrying out CLIA is vested in the Secretary of Health and Human Services (HHS) under Section 353 of the Public Health Service Act, as amended. The new section 353 required the Department of HHS to establish certification requirements for any laboratory that performs tests on human specimens, and certify through issuance of a certificate that those laboratories meet the certificate requirements established by HHS.

The Secretary of HHS then delegated to HCFA the responsibility for the implementation of CLIA, including laboratory registration, fee collection, surveys, surveyor guidelines and training, enforcement, approval of Proficiency Testing (PT) providers, accrediting organizations and exempt states. The Centers for Disease Control and Prevention (CDC) has been responsible for test categorization, development of technical standards, and CLIA studies. Within HCFA, the Division of Outcomes and Improvements, within the Family and Children's Health Program Group, under the Center for Medicaid and State Operations (within HCFA) has the responsibility for implementing the CLIA program.

FEDERAL AGENCIES RESPONSIBLE FOR THE IMPLEMENTATION OF CLIA

Department of Health and Human Services (DHHS)
The Secretary

Health Care Financing Administration (HCFA)	**Center for Disease Control & Prevention (CDC)**	**Food and Drug Administration (FDA)**
Medicare **Medicaid** **CLIA**		
Regional Offices (10 Regions) **Region VI – Dallas, TX**	**Clinical Laboratory Improvement Advisory Committee (CLIAC)**	
Region VI – States **Arkansas Louisiana** **New Mexico Oklahoma** **Texas**		

Summary of Agency Responsibilities Under CLIA

Federal Agencies With Responsibilities Under CLIA

In order for FDA, CDC and HCFA to carry out the different functions of CLIA, the agencies entered into interagency agreements allocating responsibilities and funding.

HCFA Responsibilities:

- Approve and monitor Proficiency Testing programs;
- Approve and monitor accrediting organizations for deemed status;
- Approve and monitor State programs; granting their laboratories exemption from CLIA;
- Develop administrative regulatory requirements;
- Implement new and revised regulatory requirements;
- Develop guidelines and survey process for surveyors;
- Develop and support a data system for information collection, analysis and reporting requirements;
- Collect fees, enroll and certify laboratories;
- Survey laboratories, ensure compliance, and take enforcement action;
- Develop, implement, and monitor the CLIA budget;
- Design and conduct training sessions for CLIA surveyors; and
- Ensure fiscal solvency of the CLIA Program which is the only fee-funded government program

CDC Responsibilities:

- Measure effectiveness of CLIA through analysis and research;
- Manage the Clinical Laboratory Improvement Advisory Committee (CLIAC);
- Develop and revise technical regulatory requirements;
- Collaborate with HCFA to:
  - Develop standards, policies and guidelines
  - Evaluate State programs for approval
  - Evaluate accrediting organizations for deemed status
  - Monitor and evaluate PT programs

FDA Responsibilities:

- Categorize tests;
- Approve tests for waiver, PPMP;

NOTE: *Under the CLIA regulations issued in 1992, the FDA was to categorize new commercial test systems as part of the 510(k) and PMA approval process. Due to financial and resource constraints, the FDA was unable to implement these tasks in the early 1990s. CDC was to categorize all in-use test systems and non-commercial test systems on request from anufacturers, users, or developers of the test system.*

Manufacturers and Congress have expressed concern that having both the CDC and FDA participate in product reviews creates "confusion, and duplication of effort". Currently, HHS is working with CDC and FDA in transitioning the responsibility for test categorization to FDA.

What is CLIA-88?

CLIA is the Clinical Laboratory Improvement Amendments of 1988. Congress passed CLIA-88, as a means for the Secretary of Health to develop comprehensive, quality standards for all laboratory testing to ensure the accuracy, reliability and timeliness of patient test results regardless of where the test was performed. A laboratory is defined as any facility which performs laboratory testing on specimens derived from humans for the purpose of providing information for the diagnosis, prevention, treatment of disease, or impairment of, or assessment of health. CLIA is a user fee funded government program; therefore, all costs of administering the program must be covered by the regulated facilities. Facilities that do not accept Medicare or Medicaid or only accept cash, or provide free laboratory testing must be certified under CLIA. It is the act of performing a laboratory test that defines the requirement of certification and not how the test is paid for. CLIA is payment neutral.

The final CLIA regulations were published on February 28, 1992 and were based on the complexity of the test method; thus, the more complicated the test, the more stringent the requirements. Three categories of tests have been established: waived complexity, moderate complexity, including the subcategory of provider-performed microscopy (PPM), and high complexity. CLIA specifies quality standards for proficiency testing (PT), patient test management, quality control, personnel and quality assurance.

Data indicates that CLIA has improved the quality of testing in the United States. The total number of quality deficiencies has decreased approximately 40% from the first cycle of laboratory surveys to the second cycle of surveys. Current PT review data concurs with these earlier findings. Due to the educational value of PT in laboratories, CLIA-88 continues to address initial PT failures with an educational, rather than punitive, approach.

Background

Prior to CLIA, HCFA regulated laboratories under two federal programs: Medicare/Medicaid and CLIA'67. HCFA had two Memoranda of Understanding (MOUs):

- In 1979 (revised 1987) an MOU agreement was signed between HCFA and the Centers for Disease Control (CDC) for provision of scientific and technical expertise on questions relating to advances in instrumentation, new technology, proficiency testing, and cytology services. In addition, prior to 1979, CDC had the responsibility for the regulation of CLIA-67 licensed laboratories. In 1979, HCFA became responsible for the regulation of these laboratories.
- In 1980, an MOU was signed between HCFA and the FDA (Food and Drug Administration) for the provision of technical assistance concerning blood bank services. HCFA assumed the responsibility for the inspection of Registered Blood Establishments that also participate in Medicare. These include transfusion facilities that were located in accredited hospitals either to collect and/or transfuse whole blood, packed cells, and/or other blood components in emergency situations.

These arrangements are longstanding and are based on department policy to coordinate activities and reduce duplicate inspections.

Legislative History

CLIA-67; Clinical Laboratory Improvement Act of 1967 [P.L. 90-174]:

To implement CLIA-67, section 5(a) Part F of title III of the Public Health Service (PHS) Act (42 U.S.C. 262-3) was amended by the changing the title to read: "Licensing — Biological Products and Clinical Laboratories" and by adding section 353 (42 (U.S.C.) 263). Section 353 regulated any laboratory engaged in interstate commerce, that is, soliciting or accepting (directly or indirectly) any specimen for laboratory examination or other laboratory procedures and required CLIA-67 licensure. Laboratories were given a full, partial, or exempt CLIA-67 license, depending on the scope of laboratory testing. Regulations included Applicability; License – Application & Renewal; Quality Control; Personnel Standards; Proficiency Testing; Accreditation; General Provisions; and Sanctions.

Medicare/Medicaid; Independent and Hospital Laboratories;

Only independent and hospital laboratories seeking Medicare/Medicaid reimbursement were regulated under Title XVIII and Title XIX of the Social Security Act. Each facility type had their own regulations to follow.

Medicare/Medicaid/CLIA-67 Regulations: August 5, 1988- Proposed [March 14, 1990 – final and effective 09/01/90]:

In April 1986, a study [Final Report on Assessment of Clinical laboratory Regulations] on clinical laboratories recommended that HHS review the existing regulations to determine how to improve the assurance of quality laboratory testing and achieve program uniformity.

The August, 1988, proposal sought to recodify the regulations for these programs [Hospital laboratories, Section 1861(e) of the Social Security Act (SSA); Independent laboratories, 1861(s)(11) and 1861(s)(12) and (13); CLIA-67, Section 353 of the Public Health Service (PHS) Act [42 U.S.C. 263(a)] interstate commerce; Medicaid, Section 1902(a)(9)(C) of the SSA] into a new Part 493 in order to simplify administration and unify the health and safety requirements for all programs as much as possible.

CLIA-88:

Beginning in 1987, a series of newspaper and magazine articles were published on the quality of laboratory testing. Also, simultaneously television programs were aired concerning the number of laboratories that were not subject to either federal or state regulations. Congress held hearings in 1988 and heard testimony from "victims"of faulty laboratory testing. Specific concerns were raised about the validity of cholesterol screening and the accuracy of Pap smear results.

Section 4064 of the Omnibus Budget Reconciliation Act of 1987 [OBRA-87 – Public Law 100-203], enacted on December 22, 1987, amended Section 1861(s)(11) to require physician offices that performed more than 5000 tests per year to meet regulations. Laboratory testing in both physicians' offices (POLs) and rural health clinics that did not accept and perform tests on referral specimens would not be subject to these revisions because both the Medicare and CLIA statues [Section 1861(s)(11) of the Act and section 351(I) of the PHS Act] respectively preclude the regulation at this time of POLs and RHC that perform tests only for their own patients.

On October 31, 1988, Congress enacted Public Law 100-578 in response to the congressional hearings. PL 100-578 greatly revised the authority (PHS Act) for the regulation of laboratories.This law revised section 353 of the PHS Act (42 U.S.C. 263a) amending CLIA-67 by expanding the Department of HHS's authority from regulation of laboratories that only accepted and tested specimens in interstate commerce to the regulation of any laboratory that tested specimens for the diagnosis, prevention, or treatment of any disease or impairment of, or the assessment of the health of human beings.

Congress then enacted OBRA-89 (Public Law 101-239) on December 19, 1989. Section 6141 removed the provision under section 4064 of OBRA-87, which would now require certification of all laboratories performing tests. In addition, it required laboratories participating in the Medicare/Medicaid programs to comply with CLIA'88 requirements.

On February 28, 1992, the final regulations for CLIA-88 were published with an implementation date of September 1, 1992. Sections of the CLIA requirements were to be phased in allowing previously non-regulated laboratories to get used to the regulations. The regulations adding Provider-Performed Microscopy Procedures (PPMP) were published on March 24, 1995. Work is currently in progress with the CDC and HCFA to develop final CLIA regulations, which will reflect all comments received since the September 1, 1992, Federal Register publication and the development of new technologies.

CLIA Certificates

To enroll in the CLIA program, laboratories must first register by completing an application, pay their certification and/or compliance fees, and if applicable, undergoes an inspection to become certified. CLIA fees are based on the certificate requested by the laboratory (that is, waived, PPM, accreditation, or compliance) and the annual test volume and types of testing performed. Waived and PPM laboratories may apply directly for their certificate as they aren't subject to routine inspections.

Those laboratories which must be surveyed routinely; i.e., those performing moderate and/or high complexity testing, may choose to meet CLIA requirements through HCFA or their agent (State Survey Agency) or an approved, private accrediting organization. The HCFA survey process is outcome oriented and utilizes a quality assurance focus and an educational approach to assess compliance.

Process Overview

A laboratory must obtain CLIA certification for any onsite laboratory testing. A CLIA application, form HCFA-116 can be obtained from either a HCFA Regional Office or State Survey Agency. Internet address: www.hcfa.gov/medicaid/clia/cliahome.htm.

The laboratory must complete the HCFA-116 (and any other additional information/forms that the State Survey Agency or Regional Office requests) and return the packet to the State Survey Agency. The HCFA-116 information is then entered into the CLIA data system. The date of the data entry becomes the participation date (the first day that testing may begin). A laboratory can not begin patient testing until a CLIA Certificate has been obtained. Laboratory billing for Medicare and/or Medicaid can not be any earlier than the participation date.

The HCFA Data System

The HCFA Data System maintains files on all CLIA certificate. It contains the Online Survey Certification and Reporting (OSCAR) System; the Online Data Input and Edit (ODIE) System; and the CLIA Data Base. The CLIA database maintains and stores data pertinent to the HCFA-116, CLIA certificate history, and accounting information.

The OSCAR/ODIE database maintains and stores data for surveys and proficiency testing results, plus generates reports based on data held in all three systems. All certificates and fee coupons are generated and issued through the HCFA Data System.

Fee coupons are mailed one (1) year prior to the expiration date of Certificate of Compliance renewals; fee coupons are mailed six (6) months prior to the expiration date of Certificate of Waiver and PPMP Certificate renewals. Certificates (if fees have been paid in full) are mailed one (1) month prior to the expiration date of a current certificate. Replacement certificates can be obtained from the Regional Offices.

If after two rebills a laboratory has not paid their CLIA fees, the HCFA data system automatically terminates the CLIA certificate. This information is sent to Medicare and Medicaid and a laboratory will not be paid for Medicare and Medicaid laboratory services after the certificate expiration date.

Certificate of Waiver or PPMP Certificates

Once the State Agency or Regional Office has entered the HCFA-116 into the system, a fee coupon is generated the next day and mailed. A flat fee is issued for a Certificate of Waiver ($150) and a Provider-Performed Microscopy

Procedures (PPMP) certificate ($200). Payment must be sent to a bank lock-box in Atlanta, Georgia. Upon receipt of payment, the payment is credited to the laboratory's account and authorization is sent to the HCFA contractor to issue and mail the certificates. Both certificate types are renewed every two years.

Certificate of Compliance (COC) – Certificate of Accreditation (COA)

If a laboratory requests a COC (survey by the State Survey Agency) or COA (survey by a private accrediting agency), the process is slightly different. The HCFA-116 data is entered into the data system, indicating either a COC or COA. If the application is for a COA, the laboratory will be assessed a user fee for a Registration Certificate and accreditation/validation user fee. This fee is paid by all accredited facilities whether they receive a Validation Survey or not.

Note: *The Validation Fee is 5% of the compliance (survey) fee if the State Survey Agency had conducted the survey. This fee covers the cost of Validation Surveys conducted by the State Survey Agency.] In addition, the State Survey Agency may request confirmation of accreditation status. If the application is for a COC, the laboratory will be issued a user fee for a Registration Certificate and the compliance (survey) fee.*

Payment must be sent to the bank lock-box in Atlanta, Georgia. Upon receipt of payment, the payment is credited to the laboratory's account and authorization is sent to the HCFA contractor to issue and mail the Registration Certificate. The Registration Certificate registers a laboratory and allows them to begin testing. It speaks nothing to the quality of laboratory testing. This certificate is good for two years or until a survey has been completed. This two-year time frame allows the State Survey Agency to conduct an onsite survey to assess facility compliance. It also provides HCFA the time to verify with the accreditation agency that the facility is actually accredited and a survey has been conducted.

If a laboratory applies for a COC, the State Survey Agency will contact the laboratory to set up a survey date for the initial survey. Surveys cannot be performed until the compliance fee has been paid. The survey is usually performed 3 – 6 months after the laboratory's registration certificate effective date. The initial survey date establishes the "Effective Date of Compliance" and will establish future survey dates (recertification). Upon completion of the survey, the survey information is entered into the data system and a fee coupon is generated for the issuance of the Certificate of Compliance. Upon receipt of payment, the HCFA contractors prepare and mail out the certificate.

If a laboratory applies for a COA, the survey is coordinated between the laboratory and accreditation agency. Once the survey has been completed, the accreditation agency will enter this data into the CLIA database. This verifies that the laboratory is actually accredited and also establishes the "Effective Date of Accreditation". Fees and certificates will be issued based on this date and renewed every two years. The Certificate of Accreditation is issued upon receipt of the appropriate certificate/validation fee

Validation/Complaint Investigations

Validation surveys are conducted to assess a continued deemed status of an accreditation agency under CLIA. Complaint investigations are conducted to determine the validity of the complaint and if any CLIA conditions are not met. HCFA authority to conduct validation and complaint surveys is found in 42 Code of Federal Regulations (CFR) Section 493.563. If HCFA should conduct a validation inspection, the laboratory must:

- Allow the accreditation agency to release to HCFA a copy of its most recent inspection and related correspondence;
- Allow HCFA or its agent to conduct the survey;
- Provide HCFA or its agent full access to the facility, equipment, materials, records and information and provide copies of information requested during the survey process; and
- Allow HCFA to monitor correction of any deficiencies found through the inspection process.

The basis for HCFA surveys is the outcome-oriented survey process. The survey may be either comprehensive (reviewing all CLIA Conditions) or focused (reviewing a specific condition or conditions). If HCFA or their agent substantiates a complaint allegation and finds condition-level deficiencies, then a full inspection of the laboratory is conducted.

What is the Accrediting Agencies' Relationship to HCFA?

Accrediting Agencies (AA), i.e., ASHI, are private, non-profit accreditation organizations, with requirements that are equal to or more stringent than the CLIA condition-level requirements, which have applied for deeming authority for CLIA. The AA must submit their requirements to HCFA for evaluation against the CLIA requirements. HCFA, together with CDC, evaluates the AA requirements and compares them to the CLIA condition-level requirements and administrative policies. If these federal agencies agree that the AA requirements are equal to or are more stringent than CLIA, the AA is approved as a deeming authority for a period of 2 – 6 years. This approval is published in the Federal Register, with the publication date as the effective date of the "deeming authority" or approval. Laboratories granted accreditation by an approved agency are deemed to meet the CLIA requirements, however, accredited laboratories must also meet the CLIA requirements.

At the time of the evaluation, HCFA and the AA enter into agreements as to how the administrative aspects of CLIA will be carried out between HCFA and the AA. The AA agrees to maintain the CLIA database with current information as to total volumes, specialties, subspecialties and demographic information on their laboratories. However, changes regarding laboratory ownership, name, location and director and technical supervisor (moderate and high complexity labs only) must be reported by the laboratory within 30 days directly to HCFA or the State Agency who will notify the carrier or fiscal intermediary (FI). The AA has no relationship or reporting responsibility to the carriers or FI's.

HCFA has a regulatory responsibility to monitor the AA performance and consider this performance in the reapproval process. As a part of this monitor, HCFA is authorized by statute to perform validation surveys. HCFA regulations authorize 5% of AA surveys to be validated. This means that within 90 days of the AA survey, HCFA or HCFA's Agent (SA) will perform a CLIA survey and assess the laboratory's compliance with the CLIA regulations. The results of the survey are

reported to HCFA Central Office who conducts a comparison of the validation and AA surveys for agreement, and determines a disparity rate. By regulation, the disparity rate cannot exceed 20%, or a full deeming authority review is initiated. Based on the validation comparison evaluation, HCFA provides Congress with an annual report of the validation survey results for all AAs.

AAs have no authority for enforcement of CLIA sanctions. They have their own enforcement and/or sanction protocols. Although HCFA maintains a Proficiency Testing (PT) database, AAs are required to monitor PT performance and take appropriate action as agreed during the AA's review and approval process.

What are the Accredited Laboratories Responsibilities?

Under 42 CFR 493, Subpart E, sections 493.551 to 493.575, HCFA outlines the requirements and responsibilities of Accreditation [and Exemption (under an approved State Laboratory Program)] under the CLIA program. Section 493.551 states that HCFA may deem (grant deemed status) a laboratory to meet all applicable CLIA program requirements through accreditation by a private nonprofit accreditation program if certain conditions are met:

- The regulations of the accreditation agency are equal to or more stringent than CLIA condition-level requirements and that the accredited laboratory must be in compliance with the CLIA condition-level requirements if inspected by HCFA and/or its agents (State Survey Agency).
- The accreditation program is approved under HCFA. ASHI received their HCFA approval on November 3, 1994.
- The laboratory must allow the release of information to HCFA by the accreditation program and permit HCFA inspections.

In addition to meeting accreditation standards, accredited laboratories must comply with all the CLIA regulations. Accreditation does not exclude a laboratory from meeting the CLIA regulations. To meet CLIA requirements through accreditation, a laboratory must:

- Treat proficiency testing samples in the same manner as patient sample;
- Meet notification requirements under section 493.63:
  - Notify HCFA and the approved accreditation program within 30 days of any changes in ownership, name, location, and/or director;
  - Notify the accreditation program no later than 6 months of performing any addition of a test or examination within a specialty or subspecialty that is not included in the laboratory's accreditation, so that the accreditation organization can determine compliance;
  - HCFA also requires notification of any additions and deletions of tests so that a laboratory's fees can be reassessed to reflect current status and a new certificate of accreditation can be issued.
- Comply with the requirements of the approved accreditation program;
- Permit random sample validation and complaint inspections;
- Permit HCFA to monitor the correction of any deficiencies found through HCFA inspections;
- Authorize the accreditation program to release to HCFA the laboratory's inspection findings for validation or complaint investigations;
- Authorize the Proficiency Testing program to release the laboratory's proficiency testing results to HCFA;
- Obtain a Certificate of Accreditation as required in subpart D and pay the applicable fees as required in subpart F of the CLIA requirements.
- Accept a full HCFA review to determine compliance (42 CFR Section 488.11), if a laboratory fails to meet the requirements listed above (failure to meet accreditation requirements) or in the event of a non-compliance determination resulting from HCFA validation or complaint inspection. The laboratory may be subject to suspension, revocation, limitation of the laboratory's certificate of accreditation or certain alternative sanctions and suspension of Medicare and/or Medicaid payments.
  NOTE: *More specific information can be found in 42 CFR 493.61 of the CLIA regulations.*

What is HCFA's perspective on evaluation of Proficiency Testing, Quality Control and Quality Assurance?

Proficiency Testing (PT)

Accredited laboratories must permit the PT agency to release their results and interpretations to the AA. Any laboratory that refuses to allow this release of results is no longer deemed to meet the CLIA conditions and will be subject to full review by HCFA. Likewise, if an accredited laboratory has demonstrated unsuccessful PT, the AA must notify HCFA of the PT results and the actions taken by the AA within 30 days of the initiation of such action(s). HCFA may, on the basis of the notification take an adverse action against the laboratory.

Any laboratory, which demonstrates unsuccessful performance (2 consecutive or 2 out of 3 unsatisfactory testing events), is subject to sanction action by the accreditation organization. Each deemed AA must specify the actions it takes to ensure the laboratory corrects the cause(s) of the PT failures. The AA action must be equivalent to those of HCFA. Current HCFA policy requires the laboratory to undertake corrective action of technical assistance and training for its first unsuccessful performance. The laboratory must demonstrate satisfactory (80% or better) performance on the next PT event.

If the laboratory performs unsatisfactorily in the next event(s), (the second unsuccessful), the laboratory is required to stop testing in the analyte(s), specialty, or subspecialty of failure and demonstrate two consecutive satisfactory events before it can resume testing.

During a CLIA survey, part of evaluating a laboratory's PT performance includes an evaluation of any unacceptable results(s) and the laboratory's corrective action. The surveyor looks for documentation to assure the laboratory has reviewed quality control, calibration, instrument maintenance, corrective action for out-of-control results, test performance, and adherence to the laboratory's policies and procedures in determining the corrective action needed. The laboratory is also required to monitor the corrective action for effectiveness through Quality Assurance.

For ungraded results, the laboratory should evaluate their results against the expected results and determine if they would have performed satisfactorily. Documentation of this evaluation must be maintained for two years. If a laboratory is enrolled in PT for unregulated analytes, this will meet the Quality Assurance requirements to assure accuracy twice a year. During a survey, the surveyor will assure there has not been two consecutive ungraded events, and if there has been, the surveyor reviews the laboratory's performance.

The AA [see section 493.557(a)(12)] must report accredited laboratories that demonstrate unsuccessful performance, for regulated analytes listed in subpart I, to HCFA. Any laboratory found to have referred PT samples to another laboratory for testing must have its accreditation denied and HCFA must be notified of the denial. Referral of PT samples requires HCFA, by statue, to revoke the laboratory's CLIA certificate for a minimum of one year. HCFA has no discretion regarding PT referral.

The purpose of PT is to provide a snapshot in time of the laboratory's quality. PT samples should be handled in the same manner as patient samples. The laboratory should perform no special instrument maintenance nor utilize special personnel when testing PT samples. PT provides an indication of the quality of patient testing and offers the laboratory an opportunity to assess its Quality Control (QC) and Quality Assurance (QA) activity. Unsuccessful PT results may be indicators that QC or QA activity needs revision, which can be the case as instruments age, new instruments are placed into service, new employees hired or other changes occur which may affect quality.

PT participation and performance is intended to be educational and not punitive. However, if a laboratory demonstrates unsuccessful; performance in 2 consecutive or 2 out of 3 testing events, the causative problems have existed for 8-12 months without identification and correction through a laboratory's QA process. This indicates a potential for jeopardizing patient testing quality and reliability.

Quality Control

Quality Control (QC) is the means by which a laboratory validates and monitors the accuracy of its patient test results on a day to day basis, and is a means, which allows the laboratory to detect error or potential sources of analytical error. However, HCFA realizes that to accomplish the outcome goal of accurate results, the QC program must be developed with all the unique laboratory factors in mind such as equipment, volume, methods, personnel, patient distribution, urgency of results, etc.. Therefore, surveyors review the laboratory's policies and procedures and QC records to assure the laboratory's stated QC goals can be realized by the established policies and procedures the laboratory has developed. Surveyors also evaluate QC results as they relate to PT results and events.

Method validation or verification is also part of QC. This does not only include in-house developed methods, but also newly implemented high complexity FDA approved methods as well as modified, moderate complexity FDA approved methods. Documentation of validation or verification needs to be maintained as long as the method is in use or two years after it is discontinued.

Quality Assurance (QA)

Quality Assurance is the system the laboratory has developed and put into place, which assures analytical accuracy and compliance with the laboratory, established policies and procedures and the CLIA regulations. The QA program should assure and document that the laboratory's stated goals for all the conditions of CLIA are met, and that when problems or outcomes (possibly adverse) are identified, they are investigated, resolved and monitored for successful resolution. The QA system ensures that the policies and procedures are appropriate for adequate monitoring and correction of problems and are effective in preventing recurrences of any identified problems.

In CLIA, the ten QA standards encompass the entire CLIA regulation. The ten QA standards are monitors of the following CLIA conditions: Patient Test Management (Subpart J), Quality Control (Subpart K), Proficiency Testing (Subpart I), Personnel (Subpart M), General Provisions (Subpart A), and Quality Assurance (Subpart P). If the laboratory has defined an effective QA system, which evaluates and monitors the ten QA standards, then all conditions of CLIA should be met.

What is HCFA's perspective on evaluation of Conditions, Immediate Jeopardy and Standard level deficiencies?

Conditions

HCFA's authority to impose sanctions on all laboratories is found in Subpart R, 42 CFR 493. The Conditions of CLIA must be met for a laboratory to be in compliance with CLIA. A laboratory that has systemic or pervasive quality problems is determined to be out of compliance with CLIA. Sentinel events can also cause a laboratory to be out of compliance with CLIA if they are of a serious nature and have far-reaching or permanent negative effects.

A laboratory can be found out of compliance under two situations: condition level deficiencies or condition level deficiencies with immediate jeopardy (IJ). When HCFA or its agent determines a laboratory to be out of compliance with the CLIA conditions, the laboratory has demonstrated failure to meet the requirements for certification. A laboratory must correct condition level non-compliance within 90 days or sanction actions will be proposed by HCFA. (42 CFR 493.1814).

Immediate Jeopardy

The same definition applies for Immediate Jeopardy (IJ) except that in this case, HCFA or its agent has determined that the laboratory's noncompliance with condition level deficiencies demonstrates a high probability that serious harm or injury to patients could occur at any time, or already has occurred and my well occur again if patients are not protected effectively from the harm, or the threat is not removed. Under 42 CFR 493.1812(a), HCFA requires the laboratory to take immediate action to remove the jeopardy. In this case, HCFA usually directs the laboratory to suspend the service until the jeopardy has been removed. A laboratory must correct IJ within 23 days or sanction action will be proposed by HCFA. (42 CFR 493.1812)

When either condition level deficiencies or condition level deficiencies with IJ are found to exist on a validation survey, the laboratory reverts to HCFA oversight until the IJ is removed and/or the conditions are met. HCFA notifies the laboratory and the AA of this situation. Once the laboratory achieves CLIA compliance, it is returned to the AA for oversight if the AA has not withdrawn or denied the laboratory's accreditation.

NOTE: *If during an accreditation survey, the AA identified IJ, the AA must notify HCFA within 10 days of a deficiency identified.*

Standards

When a laboratory has been determined to have standard-level deficient practices, this means that a requirement of CLIA has not been met, but it is not of a serious nature. A laboratory can have standard-level deficiencies yet found to be in compliance with the CLIA conditions. However, all laboratories are required to correct standard level deficiencies within 12 months or HCFA will take steps to revoke the laboratory's certificate; HCFA has no discretion on the 12-month rule.

What Does Non-compliance Signify for Accredited Laboratories

What are the consequences of denial or revocation of Accreditation?

An accredited laboratory meets CLIA requirements through the accreditation program. As noted previously, there are certain requirements a laboratory must adhere to. If for some reason, a laboratory's accreditation has been withdrawn or revoked, the laboratory retains its Certificate of Accreditation for 45 days after the laboratory receives notice from the accreditation agency or the effective date of any action taken by HCFA, whichever is the earlier date. It is the responsibility of the accrediting agency and laboratory to inform HCFA of this. The laboratory has several options which includes: change to a lower certificate type; stop laboratory testing; apply for and obtain a COC, or obtain a Registration Certificate and apply for accreditation through another accreditation organization. Once this has been determined, the certificate/billing process starts again, based on the changes.

HCFA's authority to conduct validation and complaint surveys is found in 42 CFR 493.563. Under 42 CFR 493.567, it defines the action HCFA will initiate if a laboratory refuses to cooperate and allow the survey. The laboratory may be subject to:

- full review by HCFA or its agent; and/or
- suspension, revocation, or limitation of its certificate of accreditation;

However, if a facility withdraws its prior refusal and complies with the requirements under 42 CFR section 493.563, the validation/complaint survey will resume. In additions, actions to delay or hamper the HCFA survey process result in the same outcome, as refusal of the survey. If a laboratory is found out of compliance with CLIA conditions, it is subject to the same survey and enforcement processes (principal and alternative sanctions found in 42 CFR 493.1806) applied to laboratories that are not accredited.

Revocation

HCFA may propose revocation of a CLIA certificate based on continued condition-level non-compliance which exceeds 90 days, Immediate Jeopardy which is not removed within 23 days, referral of Proficiency Testing specimens or deficiencies which are not corrected within 12 months.

There is due process prior to the revocation taking effect, and that due process differs based on the reason for revocation. The laboratory is always aware of the proposed HCFA actions and has an opportunity to respond with evidence as to why action should not be taken. The State Operations Manual sets forth HCFA's policies and procedures for due process and gives guidelines for HCFA decisions.

Once a CLIA certificate has been revoked, the owner and/or director cannot own or direct a laboratory for two years. The name of the laboratory goes on the annual CLIA Registry, which is available on the Internet at www.hcfa.gov/medicaid/clia/cliahome.htm.

Denial

HCFA may deny a CLIA Certificate of Registration, Waiver or PPM based on the information supplied in the application (HCFA-116), additional information requested, or refusal on behalf of the laboratory to submit to HCFA the requested information needed to determine compliance with CLIA requirements for a Certificate of Registration, Waiver or PPM. If, based on the application, HCFA has substantial reason to believe a laboratory could not meet the conditions of CLIA, a Certificate of Registration, Waiver or PPM can be denied, and the laboratory is notified in writing of the denial with reason for the denial. Once denied, a laboratory may resubmit an application when the reason for the denial has been remedied.

Author Index

Patrick W. Adams, MS, CHS
Ohio State University Hospital
Department of Surgery
410 W 10th Ave
N 919 Doan Hall
Columbus, OH 43210
(614) 293-8554
FAX: (614) 293-8287
E-Mail: adams-5@medctr.ohio-state.edu

Sue Bassinger
University Hospital
2211 Lomas, NE
Albuquerque, NM 87106
(505) 277-4784
FAX: (505) 277-7224

Lee Ann Baxter-Lowe, PhD, dip.ABHI
UCSF/Immunogenetics & Transplantation Laboratory
Box 0508
San Francisco, CA 94143-0508
(415) 476-6058
FAX: (415) 476-0379
E-Mail: leeannb@itsa.ucsf.edu

Ann B. Begovich, PhD
Roche Molecular Systems
1145 Atlantic Ave
Alameda, CA 94501
(510) 814-2916
FAX: (510) 522-1285
E-Mail: Ann.Begovich@Roche.com

Anne C. Belanger, MA, MT(ASCP)
Healthcare Standards Consultants
2South723 Route 59, Ste 86
Warrenville, IL 60555-1442
(630) 876-6084
FAX: (630) 876-6084
E-Mail: abelanger@msn.com

Paula Howell Blackwell, BS, CHS, MBA
10506 Bar D Trail
Helotes, TX 78023-4057
(210) 567-5697
FAX: (210) 567-4549
E-Mail: blackwell@uthscsa.edu

Cynthia E. Blanck, PhD
3714 Huntington Drive
Amarillo, TX 79109
(806) 358-1252
FAX: (806) 354-5887
E-Mail: CEBlanck@aol.com

Robert A. Bray, PhD, dip.ABHI
Emory University Hospital
Dept of Pathology, Rm F-149
1364 Clifton NE
Atlanta, GA 30322
(404) 712-7317
FAX: (404) 727-1579
E-Mail: rbray@emory.edu

Teodorica Bugawan, BS
Roche Molecular Systems
1145 Atlantic Ave
Alameda, CA 94501
(510) 814-2909
FAX: (510) 814-2910
E-Mail: teodorica.bugawan@roche.com

Mike Bunce
Oxford Transplant Center
Tissue Typing Lab
Churchill Hospital
Oxford, OX3 7LJ
United Kingdom
01865226102
FAX: 01865226162
E-Mail: mbunce@hgmp.mrc.ac.uk

Esther-Marie Carmichael, MT(ASCP), CLS, PHM
Health Care Financing Administration
Division of State Operations
75 Hawthorne Street, 4th Floor
San Francisco, CA 94105
(415) 744-3729
E-mail: ecarmichael@hcfa.gov

Mary N. Carrington, PhD, MS
NCI-FCRDC
PO Box B
Bldg 560
Frederick, MD 21702
(301) 846-1390
FAX: (301) 846-1909
E-Mail: carringt@fcrfv2.ncifcrf.gov

Pam Chapman
Emory University Hospital
HLA Lab
1364 Clifton Rd NE
Atlanta, GA 30322
(404) 712-7365

Mary Ethel Clay, MS, MT(ASCP)
University of Minnesota Medical School
420 Delaware St SE
Box 198 UMHC Mayo
Minneapolis, MN 55455
(612) 626-1905
FAX: (612) 624-5411

Myra Coppage, MS, CHS
University of Rochester Medical Center
601 Elmwood Ave
Box 8410-Surg Rm 2-8115
Rochester, NY 14642
(716) 275-0985
FAX: (716) 271-7929
E-Mail: MyraCoppage@urmc.rochester.eduer.edu

Todd Young Cooper, MT(ASCP), CHS
University of Texas Medical Branch
301 University Blvd
RSH B804B
Galveston, TX 77550-0178
(409) 747-9550
FAX: (409) 747-9555
E-Mail: tcooper@utmb.edu

Deborah O. Crowe, PhD, dip.ABHI
DCI Lab
Trans Immuno, Ste 322
1601 23rd Ave S
Nashville, TN 37212
(615) 321-0212
FAX: (615) 321-4880
E-Mail: deborah.crowe@nashlab.dciinc.org

Agustin P. Dalmasso
University of Minnesota
Laboratory Medicine and Pathology
Box 198 Mayo
420 Delaware St SE
Minneapolis, MN 55455
(612) 625-9171

Julio C. Delgado
Brigham & Women's Hospital
75 Francis St
Boston, MA 02115
(617) 632-3346
FAX: (617) 632-4466

Mary L. Duenzl
Emory University Hospital
HLA Lab
1364 Clifton Rd NE
Atlanta, GA 30322
(404) 712-7365

Brian Duffy, MA, CHS
Barnes-Jewish Hospital
HLA Lab, One Barnes Plaza
St Louis, MO 63110
(314) 747-0435
FAX: (314) 362-4647
E-Mail: bdduff@aol.com

David D. Eckels, PhD, dip.ABHI
Blood Research Inst
PO Box 2178
Milwaukee, WI 53201-2178
(414) 937-6310
FAX: (414) 937-6284
E-Mail: ddeckels@bcsew.edu

Gail Eiber, MT
North Central Blood Services
Neutrophil/Platelet Serology Lab
American Red Cross
100 South Robert St
St Paul, MN 55107
(651) 291-6797
E-Mail: Eiber@usa.redcross.org

Marcelo Fernandez-Vina, PhD, dip.ABHI
American Red Cross
National Histo Lab
22 S Green Street, Box 173
Baltimore, MD 21201
(410) 328-2522
FAX: (410) 328-2967
E-Mail: vinam@usa.redcross.org

Soldano Ferrone
Dept Mico/Immunology
New York Medical College
Basic Science Bldg Rm 308
Valhalla, NY 10595
(914) 493-8481 and (914) 594-4175

Donna M. Fitzpatrick, CHS
Massachusetts General Hospital
32 Fruit St, WHT 544
Boston, MA 02114
(617) 726-3722
FAX: (617) 724-3331
E-Mail: dfitzpatrick@partners.org

Marilena Fotino, MD, dip.ABHI
Rogosin Inst
430 E 71st St
New York, NY 10021
(212) 772-6700
FAX: (212) 861-9473

Anne Fuller
University of Utah Hospital
50 North Medical Dr, AR SOM 121
Salt Lake City, UT 84132
(801) 581-3116

Zenaida P. Gantan, MD
Irwin Memorial Blood Centers
270 Masonic Ave
San Francisco, CA 94118
(415) 567-6400
FAX: (415) 775-3859

Howard M. Gebel, PhD, dip.ABHI
LSU Medical Center
1501 Kings Highway
Shreveport, LA 71130-3932
(318) 675-6112
FAX: (318) 675-6358
E-Mail: hgebel@lsuhsc.edu

Barbara Braun Griffith
Molecular Diagnostics Cor UNM Health Science Center
915 Camino de Salud NE, BMSB Rm 332
Albuquerque, NM 87131-5301
(505) 272-4783
FAX: (505) 272-9038
E-Mail: bgriffit@salud.unm.edu

F. Carl Grumet, MD
Stanford University
800 Welch Rd
Palo Alto, CA 94304
(650) 723-7976
FAX: (650) 725-4470
E-Mail: grumet@stanford.edu

Leigh Ann Guthrie
Fred Hutchinson Cancer Research Center
428 W 21st Ave
Spokane, WA 99203
(509) 624-7728
FAX: (509) 624-7728
E-Mail: lguthrie@fhcrc.org

Martin Gutierrez
6166 Montgomery Rd.
Elkridge, MD 21075
(410) 328-2974
FAX: (410) 328-9156
E-Mail: gutierrezm@usa.redcross.org

Julia A. Hackett, BS, HS(ABHI)
National Inst of Health
13276 Musicmaster Dr
Silver Spring, MD 20904
(301) 496-8852
FAX: (301) 480-0526
E-Mail: jhackett@dtm.cc.nih.gov

Amy B. Hahn, PhD, dip.ABHI
Albany Medical College Trans Immunology
47 New Scotland Ave
Rm ME524 Mail Code 62
Albany, NY 12208
(518) 262-5574
FAX: (518) 262-6274
E-Mail: hahna@mail.amc.edu

Charles William Hamrick, CHS, CLS
3057 Maplewood Pl
Escondido, CA 92027
(619) 642-4774
FAX: (619) 642-0595
E-Mail: chamrick@ucsd.edu

John A. Hansen, MD
Fred Hutchinson Cancer Research Center
1100 Fairview Ave N
PO Box 19024
Seattle, WA 98109-1024
(206) 667-5111
FAX: (206) 667-5255
E-Mail: jhansen@fhcrc.org

Leah N. Hartung
ARUP Institute for Clinical and Experimental Pathology, LLC
500 Chipeta Way
Salt Lake City, UT 84108-1211
(801) 584-5208
E-mail: hartunln@arup-lab.com

Sandra Helman, PhD, dip.ABHI
Medical College of GA
1120 Fifteenth St
BAS 1641
Augusta, GA 30912-4091
(706) 721-3311
FAX: (706) 721-2709
E-Mail: shelman@mail.mcg.edu

Patrice K. Hennessy, CHT
Ohio State University
410 W 10th Ave
N-935 Doan Hall
Columbus, OH 43210
(614) 293-8554
FAX: (614) 293-8287
E-Mail: hennessy-1@medctr.osu.edu

Nancy F. Hensel, CHS, MT(ASCP)
Nat'l Inst of Health Hematology Branch
9000 Rockville Pike
Bldg 10 Rm 7C103
Bethesda, MD 20892-1652
(301) 402-3296
FAX: (301) 496-8396
E-Mail: henseln@nih.gov

Susie E. Herbert
Ochsner HLA Lab
2606 Jefferson Hwy
New Orleans, LA 70121
(504) 842-3769
FAX: (504) 842-2357

Debra D. Hiraki, PhD
Stanford University Stanford Blood Center
800 Welch Rd
Palo Alto, CA 94304
(415) 725-4478
FAX: (415) 725-4470

Joan E. Holcomb, MS, CHS
Emory University Hospital
HLA Lab Room C184
1364 Clifton Rd NE
Atlanta, GA 30322
(404) 712-7365
FAX: (404) 712-4717
E-Mail: Joan_Bray_Holcomb@Emory.org

Kathy A. Hopkins, MPH
Johns Hopkins University
Immunogenetics Lab
2041 E Monument St
Baltimore, MD 21205
(410) 955-3600
FAX: (410) 955-0431
E-Mail: hccs@erols.com

Louise M. Jacobbi
Saturn Management Services
Legacy Donor Foundation
208 Glenwood Drive
Metairie, LA 70005
(504) 835-2767
FAX: (504) 835-2069
E-Mail: ljacobbi@aol.com

Fran Keller
UCSD Immunogenetics & Transplant
9894 Genesee Ave
Suite 101
La Jolla, CA 92037
(619) 642-4774
FAX: (619) 642-0595

Carol Kosman
Georgetown University
3970 Reservoir Rd NW
E404 Research bldg
Washington, DC 20007
(202) 687-2142

Malak Kotb
University of Tennessee
956 Court Ave, Ste A202
Memphis, TN 38163
(901) 448-5924
FAX: (901) 448-7306

Shalini Krishnaswamy
Stanford University
800 Welch Rd
Suite #3NE
Stanford, CA 94304
(415) 725-4478
FAX: (415) 725-4470
E-Mail: Shalini@cmgm.Stanford.edu

Geoffrey A. Land, PhD, HCLD
Methodist Med Center
1441 N Beckley Ave
Dallas, TX 75203
(214) 947-3584
FAX: (214) 947-3598
E-Mail: galand@flash.net

Lauralynn K. Lebeck, PhD, MS, dip.ABHI
University of California San Diego
9894 Genesee Ave, #101
La Jolla, CA 92037
(858) 642-4774
FAX: (858) 642-0595
E-Mail: llebeck@ucsd.edu

Jar-How Lee, PhD, dip.ABHI
One Lambda, Inc Res Dept
21001 Kittridge St
Canoga Park, CA 91303-2801
(818) 702-0042
FAX: (818) 702-6904
E-Mail: jlee@onelambda.com

M. Sue Leffell, PhD, ABMLI, dip.ABHI
Johns Hopkins University
2041 E Monument St
Baltimore, MD 21205-2222
(410) 614-8976
FAX: (410) 955-0431
E-Mail: msleffel@jhmi.edu

William M. LeFor, PhD
LifeLink Foundation, Inc.
Trans Immuno Lab
409 Bayshore Blvd
Tampa, FL 33606
(813) 253-3866
FAX: (813) 254-3367
E-Mail: lefor@lifelinkfound.org

Nufatt Leong
University of Rochester Med Center Tissue Typing Lab
601 Elmwood Ave
Box Surgery
Rochester, NY 14642
(716) 275-0985
E-Mail: nufatt_leong@urmc.rochester.edu

Jimmy Loon
One Lambda, Inc
21001 Kittridge St
Canoga Park, CA 91303
(818) 702-0042
FAX: (818) 702-6904
E-Mail: jloon@onelambda.com

David F. Lorentzen
University of Wisconsin Hospitals & Clinic
600 Highland Ave
HLA - Moleculor Diagnostics
Madison, WI 53792
(608) 263-8808
FAX: (608) 263-1568
E-Mail: df.lorentzen@hosp.wisc.edu

Patrizia Luppi
University of Pittsburgh Histocompatability Center
Rangos Research Center
3460 Fifth Avenue
Pittsburgh, PA 15213-3205
(412) 692-6570
FAX: (412) 692-5809

Prema R. Madyastha, PhD
Department of Pediatric Endocrinology
Medical University of South Carolina
171 Ashley Avenue
Charleston, SC 29401
(843) 792-6807
E-Mail: madyaspr@musc.edu

Maureen P. Martin
SAIC-Frederick, NCI-RCRDC
PO Box B
Bldg 560, Rm 21-46
Frederick, MD 21702
(301) 846-1309
FAX: (301) 846-1909
E-Mail: martinm2mail.ncifcrf.gov

Paul Joseph Martin, MD
Fred Hutchinson Cancer Center
1100 Fairview Ave. N, 02-100
Seattle, WA 98109
(206) 667-4798
FAX: (206) 667-5255

Jeffrey M. McCormack, PhD, dip.ABHI
DigiScript, Inc.
381 Mallory Station Road, Suite 210
Franklin, TN 37067
(615) 778-0780
FAX: (615) 778-0781
E-Mail: jmccormack@digiscript.com

Chris Mcfarland
Fred Hutchinson Cancer Research Center
1100 Fairview Ave N
PO Box 19024
Seattle, WA 98109-1024
(206) 667-4362
FAX: (206) 667-5892

Arvind K. Menon, MS
The Rogosin Inst Immuno & Trans Center
430 E 71st St
New York, NY 10021
(212) 772-6700
FAX: (212) 861-9473

Eric Mickelson
Fred Hutchinson Cancer Center
1100 Fairview Ave N
PO Box 19024
Seattle, WA 98109-1024
(206) 667-4922
FAX: (206) 667-5255
E-Mail: emickels@fhcrc.org

Derek Middleton
Belfast City Hospital
Belfast, BT9 7TS
Northern Ireland
44 1232 263676
FAX: 44 1232 263881
E-Mail: derek.middleton@bll.n-i.nhs.uk

E.L. Milford, MD
Brigham and Women's Hospital and New England Organ Bank
Tissue Typing Lab
75 Francis St
Boston, MA 02115

Aloke Mohinen
American Red Cross
National Histo Lab
22 S Green Street, Box 173
Baltimore, MD 21201
(410) 328-2522
FAX: (410) 328-2967

Priscilla V. Moonsamy
Roche Molecular Systems
1145 Atlantic Ave
Alameda, CA 94501
(510) 814-2953
FAX: (510) 522-1285
E-Mail: Priscilla.Moonsamy@Roche.com

Beverly Muth
American Red Cross
22 S Greene St
Box 173
Baltimore, MD 21201
(410) 328-2968
FAX: (410) 328-9156

Debra K. Newton-Nash, PhD
Blood Center of Southeastern Wisconsin
PO Box 2178
Milwaukee, WI 53201-2178
(414) 937-6222
E-Mail: debra@smtpgate.bcsew.edu

Afzal Nikaein, PhD
TX Medical Specialty, Inc
7777 Forest Lane
12A South
Dallas, TX 75230
(972) 566-5794
FAX: (972) 566-3897
E-Mail: nikaein@cs.com

Brenda Nisperos
Fred Hutchinson Cancer Center
1124 Columbia St
Seattle, WA 98104
(206) 292-5768
FAX: (206) 667-5285

Charles G. Orosz, PhD
Ohio State University
1654 Upham Dr
357 Means Hall
Columbus, OH 43210
(614) 293-3212
FAX: (614) 293-4541
E-Mail: orosz-1@medctr.osu.edu

John W. Ortegel
Dept of Internal Medicine
Section of Pulmonary & Critical Care Medicine
Rush Presbyterian/St. Luke's Med Center
Chicago, IL 60612
(312) 942-2745
FAX: (312) 563-2157
E-Mail: jortegl@rush.edu

Lori Dombrausky Osowski, MS, CHS
American Red Cross
National Histocompatability Lab
22 S Greene St Box 173
Baltimore, MD 21201-1595
(410) 328-2973
FAX: (410) 328-2967
E-Mail: osowskil@usa.redcross.org

Sandra Pearson, MT(ASCP)
Health Care Financing Administration
CLIA Program
1301 Young Street, Rm 833
Dallas, TX 75202
(214) 767-4414
E-mail: spearson@hcfa.gov

Herbert A. Perkins, MD
Blood Centers of the Pacific
270 Masonic Ave
PO Box 18718
San Francisco, CA 94118-4496
(415) 749-6652
FAX: (415) 921-6184
E-Mail: hperkins@pacbell.net

Donna L. Phelan, BA, CHS, MT(HEW)
Barnes-Jewish Hosp Labs
One Barnes Plaza
St Louis, MO 63110
(314) 362-6527
FAX: (314) 362-4647
E-Mail: dlphelan@aol.com

Diane J. Pidwell, PhD MT(ASCP) dipABHI
12402 Old Harmony Landing
Goshen, KY 40026
(502) 587-4373
FAX: (502) 587-4504
E-Mail: diane.pidwell@jhhs.org

Marilyn S. Pollack, PhD, dip.ABHI
University of Texas Health Science Center
7703 Floyd Curl Dr
Dept. of Surgery
San Antonio, TX 78229-3900
(210) 567-5697
FAX: (210) 567-4549
E-Mail: pollack@uthscsa.edu

Lisa Ratner-Rothstein
Brigham & Women's Hospital Tissue Typing Lab
75 Francis St
Boston, MA 02115
(617) 732-5872

Elaine F. Reed, PhD, dip.ABHI
UCLA Immunogenetics Center
Dept. of Pathology
950 Veteran Ave
Los Angeles, CA 90095
(310) 825-7651
FAX: (310) 206-3216
E-Mail: ereed@mednet.ucla.edu

Nancy Reinsmoen, PhD, dip.ABHI
Duke University Medical Center
Box 3712
Research Park III
Durham, NC 27710
(919) 684-3089
FAX: (919) 684-9089
E-Mail: reins001@mc.duke.edu

Laura Roberts
St Francis Hospital
Histocompatibility Lab
6161 South Yale Avenue
Tulsa, OK 74136
(918) 494-6569
FAX: (918) 494-1603
E-Mail: ldroberts@saintfrancis.com

Anthony L. Roggero, CHS, CHT, MT(ASCP)
Louisianna State Universityersity Medical Center
1501 Kings Hwy
Rm 3-204
Shreveport, LA 71130
(318) 675-6115
FAX: (318) 675-4243
E-Mail: arogge@lsumc.edu

William A. Rudert, MD, PhD
University of Pittsburgh
3705 Fifth Ave
Pittsburgh, PA 15213
(412) 692-6572
FAX: (412) 692-5809

Nancy Setsuko Sakahara, BS, MT(ASCP)
Irwin Memorial Blood Centers Scientific Services
270 Masonic Ave
San Francisco, CA 94118
(415) 567-6400 x446
FAX: (415) 775-3859

Patti Samuels Saiz, CHS, CHT
Pinehurst Apartments
12301 N. McArthur # 407
Oklahoma City, OK 73142
(405) 271-7647
FAX: (405) 271-7332
E-Mail: PLSaiz@aol.com

Doreen Sese
Brigham & Women's Hospita
75 Francis St
Boston, MA 02115
(617) 738-4650
FAX: (617) 566-6176

Alan R. Smerglia
Cleveland Clinic Allogen Labs
9500 Euclid Ave
C100
Cleveland, OH 44195-5131
(216) 444-6583
FAX: (216) 444-8261
E-Mail: ars@tt.ccf.org

Anajane G. Smith, MA
Fred Hutchinson Cancer Center
1100 Fairview Ave N
PO Box 19024
Seattle, WA 98109-1024
(206) 667-5743
FAX: (206) 667-5285
E-Mail: asmith@fhcrc.org

Peter Stastny, MD
UT Southwestern Medical Center
5323 Harry Hines Blvd
MC 8886
Dallas, TX 75235
(214) 648-3556
FAX: (214) 648-2949
E-Mail: ps@sta08.swmed.edu

Lori Steiner
Roche Molecular Systems
1020 Atlantic Ave
Alameda, CA 94501
(510) 814-2924
FAX: (510) 814-2910
E-Mail: Lori.Steiner@roche.com

Linda Stempora
5735 S. Meade
Chicago, IL 60638
011-41-794757787
E-Mail: lstempora@yahoo.com

Dod Stewart, BS, CHS
Ochsner HLA Lab
2606 Jefferson Hwy
New Orleans, LA 70121
(504) 842-3027
FAX: (504) 842-2357
E-Mail: dstewart@ochsner.org

Douglas Michael Strong, PhD, MT(ASCP), BCLD
Puget Sound Blood Center
921 Terry Ave
Seattle, WA 98104
(206) 292-1889
FAX: (206) 292-8030
E-Mail: dmstrong@psbc.org

Nicole Suciu-Foca, PhD, MS, BS
Columbia University
630 W 168th St
Dept of Pathology PS 14-401
New York, NY 10032
(212) 305-6941
FAX: (212) 305-3429
E-Mail: ns20@columbia.edu

Anat R. Tambur, DMD, PhD, dip.ABHI
6720 Karlov Ave.
Lincolnwood, IL 60646
(312) 942-2054
FAX: (312) 942-6965
E-Mail: atambur@rush.edu

Gary A. Teresi, MT, CHS
Allogen Laboratories
Cleveland Clinic Found-C100
9500 Euclid Ave
Cleveland, OH 44195
(216) 444-0384
FAX: (216) 444-8261
E-Mail: gat@tt.ccf.org

Massimo M. Trucco, MD
University of Pittsburgh Histocompatability Center
Rangos Research Center
3460 Fifth Avenue
Pittsburgh, PA 15213-3205
(412) 692-6570
FAX: (412) 692-5809

Smita Vaidya, PhD
University of Texas Medical Branch
301 University Blvd
404 8th St Rm B804F RSH
Galveston, TX 77555-0178
(409) 747-9550
FAX: (409) 747-9555
E-Mail: smita.vaidya@utmb.edu

Anne M. Ward
9000 Harry Hines Blvd
Tissue Antigen Lab
Dallas, TX 75235
(214) 358-5022

Ken Welsh
Oxford Transplant Center Transplant Immunology
Churchill Hospital
Oxford, OX3 7LJ
United Kingdom
0111865226122
FAX: 0111865225616

Thomas M. Williams, MD, dip.ABHI
University of New Mexico Health Sciences Center
915 Camino de Salud, NE
Rm 337-BRF
Albuquerque, NM 87131-5301
(505) 272- 8059x5872
FAX: (505) 272-5186
E-Mail: twilliams@salud.unm.edu

Lisa Wilmoth-Hosey
Emory University Hospital
1364 Clifton Rd, NE
Atlanta, GA 30322
(404) 712-7365
FAX: (404) 712-4717
E-Mail: lisa_wilmoth.hosey@emory.org

Carl T. Wittwer
Flow Cytometry
Department of Pathology
University of Utah Medical Center
Salt Lake City, UT 84143
(801) 581-4737

Jin Wu
University of New Mexico
Health Sci Center-BMSB Room 308
915 Camino De Salud, NE
Albuquerque, NM 87131
(505) 272-4784
FAX: (505) 272-1950
E-Mail: JWU@medusa.unm.edu

Edmond Yunis, MD
American Red Cross
180 Rustcraft Rd
NE Region, Ste 115
Dedham, MA 02026
(781) 461-2146
FAX: (781) 461-2269

Adriana Zeevi, PhD, dip.ABHI
University of Pittsburgh Medical Center
Biomedical Sci Tower Rm W1552
Lothrop & Terr Sts
Pittsburgh, PA 15261
(412) 624-1073
FAX: (412) 624-6666
E-Mail: zeevi+@pitt.edu

Standards for Histocompatibility Testing

Adopted 4/98

A – GENERAL POLICIES

A1.000 These Standards have been prepared by the Committee on Quality Assurance and Standards of the American Society for Histocompatibility and Immunogenetics (ASHI), and have been approved by the ASHI Council and CLIA.

A2.000 These Standards have been established for the purpose of ensuring accurate and dependable histocompatibility testing consistent with the current state of technological procedures and the availability of reagents.

A3.000 These Standards establish minimal criteria which all histocompatibility laboratories must meet if their services are to be considered acceptable. Many laboratories, because of extensive experience and long-established programs of reagent procurement and preparation, will exceed the minimal requirements of these Standards.

A4.000 Certain Standards are obligatory. In these instances, the Standards use the word "must." Some Standards are highly recommended but not absolutely mandatory. In these instances the Standards use words like "should" or "recommended."

A5.000 Procedures to be used in histocompatibility testing often have multiple acceptable variations. The accuracy and dependability of each procedure must be documented in each laboratory or by published data from other laboratories. Use of the ASHI Technical Manual is highly recommended as a reference procedure manual for all laboratories.

A6.000 Some procedures have sufficient documentation of effectiveness to warrant their use in clinical service even though they are not available in or obligatory for all laboratories.

A7.000 The use of the name of the American Society for Histocompatibility and Immunogenetics as certification of compliance to these Standards may only be made by laboratories which have been accredited through the ASHI accreditation process.

B – PERSONNEL QUALIFICATIONS

B1.000 A **Director/Technical Supervisor** must hold an earned doctoral degree in a biologic science, or be a physician, and subsequent to graduation must have had four years experience in immunology or cell biology, two of which were devoted to formal training in human histocompatibility testing. Credit toward this 96 weeks can be applied at the rate of 19 weeks for each year of appropriate working experience in human histocompatibility testing. The Director must have documentation of professional competence in the appropriate activities in which the laboratory is engaged. This should be based on a sound knowledge of the fundamentals of immunology, genetics and histocompatibility testing and reflected by external measures such as participation in national or international workshops and publications in peer-reviewed journals. He/she is available on site commensurate with workload at the laboratory, provides adequate supervision of technical personnel, utilizes his/her special scientific skills in developing new procedures and is held responsible for the proper performance, interpretation and reporting of all laboratory procedures and the laboratory's successful participation in proficiency testing.

B2.000 A **General Supervisor** must hold a bachelor's degree and have had three years' experience in human histocompatibility testing under the supervision of a qualified Director/ Technical Supervisor or five years of supervised experience if a bachelor's degree has not been earned. CHS (ABHI) certification is highly recommended.

B3.000 A **Histocompatibility Technologist** must have had one year of supervised experience in human histocompatibility testing, regardless of academic degree or other training and experience. It is highly recommended that they be either CHS or CHT (ABHI) certified. The term Technician is applied to trainees and other laboratory personnel with less than one year's supervised experience in human histocompatibility testing, regardless of academic degree or other training and experience.

B4.000 The size of the staff must be large enough to carry out the volume and variety of tests required without a degree of pressure which will result in errors.

B5.000 All personnel must meet the standards which are required by Federal, State and local laws.

C – GENERAL COMMENTS AND QUALITY ASSURANCE

C1.000 Facilities

C1.100 Laboratory space must be sufficient so that all procedures can be carried out without crowding to the extent that errors may result.

C1.200 Lighting and ventilation must be adequate.

C1.300 Refrigerators and freezers must be maintained at temperatures optimal for storage of each type of sample or reagent. They must be monitored daily. Recording thermometers are recommended for mechanical refrigerators or freezers. These should be coupled to alarm systems with an audible alarm where it can be heard 24 hours a day. In laboratories where liquid nitrogen is utilized for storage of frozen cells, the level of liquid nitrogen in the cell freezers must be monitored at intervals which will ensure an adequate supply at all times. Ambient temperature and/or the temperatures of incubators in which test procedures are carried out must be monitored daily to ensure that these procedures are carried out within temperature ranges specified in the laboratory's procedure manual.

C1.400 Laboratories performing mixed lymphocyte cultures, HLA-D, or cellular Class II typing should have a laminar flow hood or other appropriately aseptic work area. Counters should be standardized according to the manufacturer's instructions at regular intervals. The incubator should be monitored daily in relation to temperature (37°C) and CO_2 concentration (5% +/- 1%) and should be appropriately humidified.

C1.500 Laboratories using radioactive materials must store radioactive materials and conduct procedures using radioactive materials in a designated section of the laboratory. Radioactive materials must be disposed of at locations designated by local institutions.

C1.600 Equipment Maintenance and Function Checks

C1.610 The laboratory must establish and employ policies and procedures for the proper maintenance of equipment, instruments and test systems by 1) defining its preventive maintenance program for each instrument and piece of equipment, and by 2) performing and documenting function checks on equipment with at least the frequency specified by the manufacturer.

C1.700 Adequate facilities to store records must be immediately available to the laboratory.

C1.800 The laboratory must be in compliance with all applicable Federal, State and local laws which relate to laboratory employee health and safety; fire safety; and the storage, handling and disposal of chemical, biological and radioactive materials.

C1.900 Computer assisted analyses must be reviewed, verified and signed by the Supervisor and/or Laboratory Director before issue.

C1.910 The computer software program used for analyses must be documented.

C2.000 Specimen Submission and Requisition.

C2.100 The laboratory must have available and follow written policies and procedures regarding specimen collection.

C2.110 The laboratory must perform tests only at the written or electronic request of an authorized person. The laboratory must assure that the requisition includes: 1) the patient's name or other method of specimen identification to assure accurate reporting of results; 2) the name and address of the authorized person who ordered the test; 3) date of specimen collection; 4) time of specimen collection, when pertinent to testing; 5) source of specimen. Oral requests for laboratory tests are permitted only if the laboratory subsequently obtains written authorization for testing within 30 days of the request.

C2.120 Blood samples must be individually labeled as to the name, or other unique identification marker for the donor and the date of collection. When multiple blood tubes are collected, each tube must be individually labeled.

C2.130 The laboratory must maintain a system to ensure reliable specimen identification, and must document each step in the processing and testing of patient specimens to assure that accurate test results are recorded.

C2.140 The laboratory must have criteria for specimen rejection and a mechanism to assure that specimens are not tested when they do not meet the lab's criteria for acceptability.

C2.200 Blood samples must be obtained using a location which does not compromise aseptic techniques. The donor's skin must be prepared by a technique which ensures minimal possibility of infection of the donor or contamination of the sample. All needles and syringes must be disposable.

C2.210 All blood samples should be handled and transported in accordance with the understanding that they could transmit infectious agents.

C2.220 The anticoagulant/preservation medium used must be shown to preserve sample viability, antigens and distributions of markers/characteristics of cells tested for the (maximum) length of time and under all the specified storage conditions the laboratory permits, on the basis of documented or published stability tests, between sample collection and testing.

C2.300 Reagents.

C2.310 All reagents must be properly labeled and stored according to manufacturers' instructions. Each serum or monoclonal antibody or typing tray must be stored at a temperature appropriate to maintaining its reactivity and specificity.

C2.320 Reagents, solutions, culture media, controls, calibrators and other materials must be labeled to indicate 1) identity and when significant, titer, strength or concentration; 2) recommended storage requirements; 3) preparation and/or expiration date and other pertinent information.

C3.000 All procedures in use in the laboratory must be detailed in a procedure manual which is immediately available where the procedures are carried out. The procedure manual must be reviewed at least annually by the Director and written evidence of this review must be in the manual. Any changes in procedures must be initialed and dated by the Director at the time they are initiated.

C4.000 Quality Assurance

C4.100 The laboratory must participate in at least one external proficiency testing program, if available, in each category for which ASHI accreditation is sought.

C4.200 If a laboratory's performance in an external proficiency testing program is unsatisfactory in any category for which ASHI accreditation is sought, the laboratory must participate in an enhanced proficiency testing program in that category until performance is deemed satisfactory.

C4.300 Proficiency test samples must be tested in a manner comparable to that for testing patient samples.

C4.400 The laboratory must, at least once each month, give each individual performing tests a characterized specimen as an unknown to verify his or her ability to reproduce test results. The laboratory must maintain records of these results for each individual.

C4.500 The laboratory must establish and employ policies and procedures, and document actions taken when 1) test systems do not meet the laboratory's established criteria including quality control results that are outside of acceptable limits; and when 2) errors are detected in the reported patient results. In the latter instance, the laboratory must promptly a) notify the authorized person ordering or individual utilizing the test results of reporting errors; b) issue corrected reports, and c) maintain copies of the original report as well as the corrected report for two years.

C5.000 Records and Test Reports.

C5.100 The laboratory must maintain a legally reproduced record of each test result, including preliminary reports, for all subjects tested for a period of two years or longer, depending on local regulations.

C5.110 These records must include log books, and at least a summary of results obtained.

C5.120 Work sheets must clearly identify the subject whose cells were tested, the typing sera which were used, the date of the test and the person performing the test.

C5.130 For each cell-serum combination, the results must be recorded in a manner which indicates the approximate percent of cells killed. The numerical codes used in the ASHI Laboratory Manual are recommended.

C5.140 Reports or records, as appropriate, should include a brief description of the specimen (blood, lymph node, spleen, bone marrow, etc.) used for testing.

C5.150 Membranes or autoradiographs from nucleic acid analysis must be retained as a permanent record.

C5.160 Records may be saved in computer files only, provided that back-up files are maintained to ensure against loss of data. It is recommended that legal advice be sought to be certain that computer files meet requirements in case of legal actions.

C5.170 For marrow transplantation, the donor must give his informed consent before blood is taken for typing and before the donor is placed on a list of donors available to be called.

C5.180 For marrow transplantation, donor records should be maintained so that donors can be rapidly retrieved according to HLA type.

C5.190 The laboratory must have adequate systems in place to report results in a timely, accurate and reliable manner.

C5.200 The report should contain:

a. The date of collection of sample.
b. The Laboratory and/or Institution's unique identifiernumber.
c. The name of the individual tested.
d. The date the individual was tested.
e. The date of the report.
f. The test results.
g. Any appropriate control value/normal ranges, where appropriate.
h. Appropriate interpretations and the signature of the Laboratory Director, or designate in his/her absence.

C5.210 The laboratory must indicate on the test report any information regarding the condition and disposition of specimens that do not meet the laboratory's criteria for acceptability.

C5.220 The laboratory must maintain permanent files of all internal and external quality control tests.

C5.230 Laboratories should have a mechanism in place for resolving any tissue typing discrepancies that may occur between laboratories.

C6.000 The Laboratory Director and technical staff must participate in continuing education relative to each category for which ASHI accreditation is sought.

C7.000 An accredited laboratory may engage another laboratory to perform testing not done by the primary laboratory. In that event, the subcontracting laboratory must be accredited by the American Society for Histocompatibility and Immunogenetics, if the testing is covered by ASHI Standards. If genetic systems not covered by ASHI Standards (ABO, RBC enzymes, etc.) are subcontracted, the subcontracting laboratory must document expertise and/or accreditation in those systems. The identity of the subcontracting laboratory and that portion of the testing for which it bears responsibility must be noted in the reports.

D – HLA ANTIGENS

D1.000 Terminology of HLA antigens must conform to the latest report of the W.H.O. Committee on Nomenclature.

D1.100 Potential new antigens not yet approved by the W.H.O. Committee must have a local designation which cannot be confused with W.H.O. terminology.

D1.200 Phenotypes and genotypes should be expressed as recommended by the W.H.O. Committee, as in the following examples:

D1.210 Single antigens: HLA-B7 (or B7 if HLA is obvious from context).

D1.211 The locus designation must always be included.

D1.220 Phenotype: HLA-A2,30; B7(Bw6), 44(Bw4); Cw5; DR1,4; DQ5,7; Dw1,w4.

D1.221 If only a single antigen is found at a locus, the phenotype may include it twice only if homozygosity is proven by family studies. Conversely, a "blank antigen" can only be assigned if proven by family studies.

D1.230 Genotype:

HLA-A2,B44(Bw4),Cw5,DR1,DQ5,Dw1/A30,B7(Bw6), Cwx,DR4,DQ7,Dw4.

D2.000 Determination of haplotypes and genotypes can only be done by family studies.

D2.100 Family studies.

D2.110 All available members of the immediate family should be typed.

D2.111 Typing for HLA-A,-B locus antigens is mandatory. Typing for HLA DR is highly recommended.

D2.112 Typing for HLA-C, -D, -DQ and/or -DP may be helpful in some situations but is not mandatory.

D2.113 Reports of HLA family studies must include haplotype assignments and an explanation of recombination when this occurs.

D2.200 Unrelated Individuals.

D2.210 The probability of possible haplotypes, given the phenotype, may be determined from known haplotype frequencies in the relevant population.

D2.220 The haplotype frequencies used should be from the most complete and reliable studies available.

D2.230 The haplotype frequencies used should be those most appropriate for the ethnic group of the subject.

D2.240 Reports of probable haplotypes based on population frequencies should clearly indicate that they were so derived.

D3.000 The laboratory must have a written policy that it follows that establishes when antigen redefinition and retyping are required.

E – SEROLOGIC TYPING – HLA CLASS I

E1.000 HLA-A locus antigens.

E1.100 The laboratory must be able to type for all HLA-A specificities which are officially recognized by the W.H.O. and for which sera are readily available.

E2.000 HLA-B locus antigens.

E2.100 The laboratory must be able to type for all HLA-B specificities which are officially recognized by the W.H.O. and for which sera are readily available.

E3.000 HLA-C locus antigens.

E3.100 Typing for C locus antigens is not mandatory.

E3.200 If C locus typing is done, the laboratory should make continuing efforts to type for all C locus antigens for which sera can be obtained.

E4.000 Serologic typing techniques – HLA Class I

E4.100 Techniques used must be those which have been established to define HLA Class I specificities optimally.

E4.200 Techniques used should employ minimal amounts of rare reagents. In general, only 1 microliter of each typing serum should be used in each serological test. When monoclonal antibodies are used, the amount should be adequate to ensure accuracy of the assay employed.

E4.300 Control sera.

E4.310 Each typing must include at least one positive control serum, previously shown to react with all cells expressing Class I antigens.

E4.311 Typing results may be invalid if the positive control fails to react as expected.

E4.320 Each typing must include at least one negative control serum. The negative control should either be one previously shown to lack antibody or should be from a healthy male with no history of blood transfusion.

E4.321 Cell viability in the negative control well at the end of incubation must be sufficient to permit accurate interpretation of results. For most techniques, viability should exceed 80%.

E4.322 In assays in which cell viability is not required, results on positive and negative controls must be sufficiently discriminatory to permit accurate interpretation of results.

E4.400 Target Cells.

E4.410 Cells may be obtained from peripheral blood, bone marrow, lymph nodes or spleen, or cultured cells.

E4.411 If the cell donor has been transfused within the previous seven days, results are acceptable only if antigens are unequivocally defined, with no more than two antigens per locus.

E4.420 Typing for HLA Class I antigens may employ mixed mononuclear cells or T-lymphocyte-enriched preparations.

E4.500 Each HLA-A,B,C antigen should be defined by at least two sera, if both are operationally monospecific. If multispecific sera must be used, at least three partially non-overlapping sera should be used to define each HLA-A,B,C antigen.

E4.600 Each monoclonal antibody used for alloantigen assignment must be used at a dilution and with a technique in which it demonstrates: 1) specificity comparable to antigen assignment by alloantisera on a well-defined cell panel or 2) specificity officially recognized by the W.H.O.

E5.000 Internal Quality Control.

E5.100 Cell panels of known HLA Class I type must be available to prove the specificity of new antibodies. The panel cells should include at least one example of each HLA antigen the laboratory should be able to define, and be from a variety of ethnic groups. Storage of at least some panel cells at 80°C or in liquid nitrogen may be necessary to insure availability of required antigens.

E5.200 Typing Sera.

E5.210 It is recommended that the specificity of typing sera obtained locally be confirmed in at least one other HLA laboratory.

E5.220 Specificity of individual sera received from other laboratories or commercial sources must be confirmed to ensure that they reveal the same specificities in the receiving laboratory.

E5.230 Each lot of new commercial typing trays must be evaluated by testing either with at least five different cells of known phenotype representing major specificities or in parallel with previously evaluated trays.

E5.300 Complement.

E5.310 Each batch of complement must be tested to determine that it mediates cytotoxicity in the presence of specific antibody, but is not cytotoxic in the absence of specific antibody.

E5.311 The test should employ multiple dilutions of complement to ensure that it is maximally active at least one dilution beyond that intended for use.

E5.312 The test should be carried out with at least two antibodies which should react with at least two different test cells and at least one cell which should not react. A strong and a weak antibody should be selected for the test, or serial dilutions of a single serum may be used.

E5.313 Complement should be tested separately for use with each type of target cell, since a different dilution or preparation may be required for optimal performance.

E6.000 External quality control.

E6.100 At least one form of external quality control must be used to ensure that local definition of HLA antigens agrees with that in other laboratories.

E6.200 The external quality control may consist of comparison of results using typing sera tested by others or typing of cells typed by others. Preferably, both approaches should be used.

E6.300 External quality controls may be carried out through local or regional arrangements and by participation in the ASHI/CAP or another equally acceptable proficiency test.

F – SEROLOGIC TYPING – HLA CLASS II

F1.000 HLA-DR Region Antigens.

F1.100 Typing for DR locus antigens is highly recommended.

F1.200 If DR locus typing is done, the laboratory must be able to type for all HLA-DR specificities for which sera are readily available, and should make continuing efforts to type for all recognized HLA-DR antigens.

F2.000 HLA-DQ Region Antigens.

F2.100 Typing for DQ locus antigens is not mandatory.

F2.200 If DQ locus typing is done, the laboratory must be able to type for all HLA-DQ specificities for which sera are readily available and should make continuing efforts to type for all recognized HLA-DQ antigens.

F3.000 HLA-DP Region Antigens.

F3.100 Typing for DP locus antigens is not mandatory.

F3.200 If DP locus typing is done, the laboratory must be able to type for those HLA-DP specificities which do not have a "w" prefix, and should make continuing efforts to type for all recognized HLA-DP antigens.

F4.000 Serologic Typing Techniques – HLA Class II

F4.100 Techniques used must be those which have been established to define HLA Class II specificities optimally.

F4.200 Techniques used should employ minimal amounts of rare reagents. In general, only 1 microliter of each typing serum should be used in each serological test. When monoclonal antibodies are used, the amount should be adequate to ensure accuracy of the assay employed.

F4.300 Control Sera.

F4.310 Each typing must include at least one positive control serum, previously shown to react with all cells expressing Class II antigens.

F4.311 Typing results may be invalid if the positive control fails to react as expected.

F4.320 Each typing must include at least one negative control serum. The negative control should either be one previously shown to lack antibody or should be from a healthy male with no history of blood transfusion.

F4.321 Cell viability in the negative control well at the end of incubation must be sufficient to permit accurate interpretation of results. For most techniques, viability should exceed 80%.

F4.322 In assays in which cell viability is not required, results on positive and negative controls must be sufficiently discriminatory to permit accurate interpretation of results.

F4.400 Target Cells.

F4.410 Cells may be obtained from peripheral blood, bone marrow, lymph nodes or spleen, or cultured cells.

F4.411 If the cell donor has been transfused within the previous seven days, results are acceptable only if antigens are unequivocally defined, with no more than two antigens per locus.

F4.420 Typing for Class II antigens usually requires B lymphocyte-enriched preparations. The proportion of B lymphocytes in each preparation must be confirmed and should usually be at least 80%.

F4.421 Separation of B lymphocytes is not required if a technique is used which distinguishes between T and B lymphocytes or in assays in which antibodies with well-defined specificity are used which only define HLA class II molecules.

F4.500 Each HLA-Class II antigen should be defined by at least three sera, if all are operationally monospecific. If multispecific sera must be used, at least five partially non-overlapping sera should be used to define each HLA-Class II antigen.

F4.510 If monoclonal antibodies are used, each DR, DQ, DP antigen should be defined by at least two antibodies with private epitope specificity or one antibody with private epitope specificity and two with public epitope specificity or at least three partially non-overlapping antibodies with public epitope specificities.

F4.600 Each monoclonal antibody used for alloantigen assignment must be used at a dilution and with a technique in which it demonstrates: 1) specificity comparable to antigen assignment by alloantisera on a well-defined cell panel or 2) specificity officially recognized by the W.H.O.

F5.000 Internal Quality Control.

F5.100 Cell panels of known HLA Class II type must be available to prove the specificity of new antibodies. The panel cells should include at least one example of each HLA antigen the laboratory should be able to define, and be from a variety of ethnic groups. Storage of at least some panel cells at -80°C or in liquid nitrogen may be necessary to insure availability of required antigens.

F5.200 Typing Sera.

F5.210 It is recommended that the specificity of typing sera obtained locally be confirmed in at least one other HLA laboratory.

F5.220 Specificity of individual sera received from other laboratories or commercial sources must be confirmed to ensure that they reveal the same specificities in the receiving laboratory.

F5.230 Each lot of new commercial typing trays must be evaluated by testing either with at least five different cells of known phenotype representing major specificities or in parallel with previously evaluated trays.

F5.300 Complement.

F5.310 Each batch of complement must be tested to determine that it mediates cytotoxicity in the presence of specific antibody, but is not cytotoxic in the absence of specific antibody.

F5.311 The test should employ multiple dilutions of complement to ensure that it is maximally active at least one dilution beyond that intended for use.

F5.312 The test should be carried out with at least two antibodies which should react with at least two different test cells and at least one cell which should not react. A strong and a weak antibody should be selected for the test, or serial dilutions of a single serum may be used.

F5.313 Complement should be tested separately for use with each type of target cell, since a different dilution or preparation may be required for optimal performance.

F6.000 External Quality Control.

F6.100 At least one form of external quality control must be used to ensure that local definition of HLA antigens agrees with that in other laboratories.

F6.200 The external quality control may consist of comparison of results using typing sera tested by others or typing of cells typed by others. Preferably, both approaches should be used.

F6.300 External quality controls may be carried out through local or regional arrangements and by participation in the ASHI/CAP or another equally acceptable proficiency test.

G – MIXED LEUKOCYTE CULTURE TESTS

G1.000 Mixed Leucocyte Culture (MLC) Test

G1.100 At the start of culture, lymphocyte viability should exceed 80%.

G1.200 Serum used in the culture medium must be screened for support of cellular proliferation and the absence of cytotoxic antibodies and must be sterile.

G1.300 MLC cultures must be incubated for the length of time shown to give appropriate proliferation.

G1.400 The negative control for each responder cell must consist of responder cells stimulated with autologous cells.

G1.500 The positive control for each responder cell must consist of either of the following: a) responder cells stimulated with cells from three or more unrelated individuals; or, b) responder cells stimulated with cells from two unrelated individuals and a pool of cells from at least three other individuals.

G1.600 In each MLC test, stimulator cells must be shown to be capable of stimulating unrelated cells.

G1.700 An MLC evaluating the potential for rejection is invalid if the cells from the potential recipient do not respond to unrelated stimulator cells. An MLC evaluating the potential for graft versus host disease is invalid if cells from a potential recipient fail to stimulate unrelated cells.

H – ANTIBODY SCREENING

H1.000 Techniques.

H1.100 A complement-dependent cytotoxic technique must be used for the detection of antibody to HLA antigens. Other techniques may be used as an adjunct to the lymphocyte-based technique if they have been demonstrated by the laboratory, or established in publications, to identify HLA-specific antibody with a specificity equivalent or superior to that of the lymphocyte-based technique or at a level appropriate for the clinical indication.

H1.120 To detect antibodies to HLA Class II antigens, a method must be used that distinguishes them from antibodies to Class I antigens.

H1.130 Techniques that detect lymphocyte-dependent antibody or test for cellular sensitization may be used to supplement the laboratory's technique that meets the requirements of H1.100.

H1.140 Techniques to identify non-HLA alloantibodies such as those using monocytes or cells from specific tissues may be used to supplement the laboratory's that meets the requirements of H1.100.

H1.150 Reports of results of antibody screening must include identification of the technique.

H1.200 Sera.

H1.210 Sera must be tested at a concentration determined to be optimal for detection of antibody to HLA antigens. The dilution(s) must be documented.

H1.220 Negative control sera must include a serum from non-alloimmunized human donor(s). Each assay must include negative control(s).

H1.230 Positive control sera should be from highly alloimmunized individuals and documented to react with HLA antigens. The antibodies must be of the appropriate isotype for each assay. Each assay must include positive control(s).

H1.300 Panel.

H1.310 The panel of HLA antigens must include sufficient panel cell donors to ensure that they are appropriate for the population served and for the use of the data.

H1.320 For assays intended to provide information on antibody specificity, documentation of the Class I and Class II phenotypes of the donors of the panel cells must be provided.

H1.330 To identify the specificity of an antibody with certainty, the laboratory should test the serum with additional cells expressing and lacking the candidate antigen and cross-reactive antigens.

H2.000 Antibody screening by complement-dependent cytotoxicity

H2.100 Positive and negative controls for the activity of complement and the viability of the test cells must be included on each tray.

H2.200 Sera must be tested undiluted.

H.2.300 Target Cells.

H2.310 Target cells may be mononuclear cells from peripheral blood, lymph nodes, spleen or cell lines or CLL.

H2.320 To detect antibodies to HLA Class II antigens, B lymphocytes, B lymphoblastoid cell lines or CLL may be used.

H2.400 The specificity of serum to be used as a reagent must be validated in other laboratories. Specificity determinations should include supporting statistical analysis.

H3.000 Antibody screening by Flow Cytometry.

H3.100 Laboratories performing assays using flow cytometry must conform to the Standards in Section Q1.000 Instrument Standardization/Calibration and in Section Q2.000 Flow Cytometric Crossmatch Technique.

H3.200 If cells pooled from multiple individuals are used for a present/not present detection of antibody, the cells used must cover the major antigen specificities or CREG. The laboratory must cite the publication used to define major antigen specificities or CREG.

H3.300 For assignment of antibody specificity, cells from a sufficient number of individuals must be used to cover appropriate specificities. To assign specificity for major antigen specificities or CREG, sufficient pools of individual cells must be used. The laboratory must cite the publication used to define major antigen specificities or CREG.

H4.000 Antibody Screening by ELISA

H4.100 Laboratories using ELISA techniques for antibody screening must conform to Standards in Sections R. Enzyme Linked Immunosorbent Assay (ELISA).

H4.200 To control for non-specific binding of antibody, each serum must be assayed in a test system which lacks HLA antigen.

H4.300 Antigens obtained from pooled cells may be used for a present/not present detection of antibody. Cells from a sufficient number of individuals must be used to cover major antigen specificities. The number of individuals must be documented.

H4.400 Sera must be tested at a concentration determined to be optimal for detection of antibody to HLA antigens. The dilution must be documented.

H4.500 The panel for HLA antigens must include sufficient panel cell donors to ensure that they are appropriate for the population served and for the use of the data.

H4.510 Antigens obtained from pooled cells may be used for a present/not present detection of antibody.

H4.520 For assays intended to provide information on antibody specificity, the manufacturer must provide documentation of the Class I and Class II phenotypes of the donors of the panel cells.

I – RENAL TRANSPLANTATION

I1.000 If cadaver donor transplants are done, personnel for the required histocompatibility testing must be available 24 hours a day, seven days a week.

I2.100 Laboratories must have a documented policy in place to evaluate the extent of sensitization of each patient at the time of their initial evaluation. (This could include testing for autoantibody, DTT reducible antibody, etc.)

I2.110 Laboratories must have a program to periodically screen serum samples from each patient for antibody to HLA antigens. Samples must be collected monthly. The laboratory must have a documented policy establishing the frequency of screening serum samples and must have data to support this policy.

I2.120 Laboratories should maintain a record of potentially sensitizing events for each patient. Serum samples should be collected and stored after each of these events for possible subsequent screening for antibody to HLA antigens and/or use in crossmatch tests.

I2.200 Antibodies of defined HLA specificity should be identified and reported.

I2.300 Studies should be performed to distinguish antibodies to HLA antigens from antibodies with other specificities.

I3.000 Crossmatching.

I3.100 Crossmatching must be performed prospectively.

I3.200 Techniques.

I3.210 Crossmatching must use techniques documented to have increased sensitivity in comparison with the standard complement-dependent, basic microlymphocytotoxicity test.

I3.220 Lymphocytotoxic or flow cytometry crossmatches must be performed with potential donor T lymphocytes and should be performed with B lymphocytes.

I3.300 Samples.

I3.310 Sera must be tested at a dilution that is optimal for each assay. For lymphocytotoxicity crossmatches, sera must be tested undiluted and should be tested at one or more dilutions.

I3.320 Sera obtained 14 days after a potentially sensitizing event should be included in a final crossmatch.

I3.400 Serum samples used for crossmatching should be retained in the frozen state for at least 12 months following transplantation.

I4.000 HLA Typing.

I4.100 Prospective typing of donor and recipient for HLA-A, B, and DR antigens is mandatory.

I4.200 Typing donor and recipient for HLA-C, DQ, DP and D antigens is optional

I5.000 Family Donors.

I5.100 All available members of the immediate family should be typed for accurate haplotype assignment.

I5.200 An MLC test may be advisable before use of a family donor. Either a one-way or a two-way MLC can be used.

I5.300 Final crossmatches performed prior to transplantation should utilize a recipient serum sample collected within the past 48 hours before transplant if the recipient has class I lymphocytotoxic antibodies (reactivity with more than 15% panel cells) or has had a recent sensitizing event (see H3.120). Otherwise, a serum collected within seven days should be used.

I5.400 A reverse lymphocytotoxicity and granulocytotoxicity crossmatch (donor serum, patient cells) is advisable in mother to child pretransplant donor specific blood transfusions.

I6.000 Cadaver Donors.

I6.100 Donors may be typed using lymphocytes from lymph nodes, spleen or peripheral blood.

I7.000 Tests to monitor the immune responsiveness of a recipient are an appropriate function for a histocompatibility laboratory. These may include, but are not limited to, the following:

I7.100 Enumeration of T lymphocytes (and subsets), B cells, NK cells and monocytes.

I7.200 Evaluation of function of T cells (cytotoxic, helper and suppressor activity), B cells (antibody production), and NK cells (cytotoxicity).

J – NON-RENAL ORGAN TRANSPLANTATION

J1.000 In cases when patients are at high risk for allograft rejection (e.g., patients with histories of allograft rejection, patients with high levels of preformed class I HLA antibodies), donors and recipients should be typed for HLA-A, B and DR antigens whenever possible.

J2.000 Patients at high risk for allograft rejection should be screened whenever possible for the presence of anti-HLA-A or B lymphocytotoxic antibodies, and for autoreactive antibodies.

J3.000 Crossmatching. See Section I3.000.

J3.100 Sera from patients at high risk for allograft rejection should be prospectively crossmatched whenever possible. Techniques with increased sensitivity (see I3.130) must be used. Crossmatch results should be available prior to transplantation of a presensitized patient.

J3.200 Final crossmatches performed prior to transplantation should utilize a recipient serum sample collected within the past 48 hours before transplant if the recipient has Class I lymphocytotoxic antibodies (determined by the laboratory's established criteria for defining positive reactivity of recipient sera against donor's unseparated cells or enriched T cells) or has had a recent sensitizing event (see I3.300). Otherwise, a serum collected within seven days should be used.

J3.300 If the patient receives a blood transfusion, has an allograft that is rejected or removed, or experiences any other potentially sensitizing event, a serum sample obtained at least 14 days post-sensitization should be used in the final crossmatch.

J3.400 Whenever possible, tissues for recipients at high risk for allograft rejection should come from crossmatch-negative donors (i.e., crossmatch with unseparated lymphocytes or enriched T-cells is less than 20% above background).

K – MARROW TRANSPLANTATION

K1.000 Histocompatibility Testing.

K1.100 HLA-A,-B,-C,-DR and -DQ typing of all available first degree relatives should be done to establish inheritance of haplotypes.

K1.120 HLA typing for HLA identical siblings (and other first degree relatives) must include adequate testing to definitely establish HLA identity. Molecular HLA typing or augmented testing (e.g., MLC, T cell precursor frequency) should be performed as appropriate for the transplant protocol and optimal donor selection.

K1.130 HLA typing for potential donors who are not first degree relatives must include molecular typing for Class II alleles at a level that is appropriate for the transplant protocol and optimal donor selection. Augmented testing (e.g., molecular typing for Class I HLA, bidirectional MLC, T cell precursor frequency) should be performed as appropriate for the transplant protocol and optimal donor selection.

K2.000 Forward and reverse lymphocytotoxicity and granulocytotoxicity crossmatch tests (patient serum, donor cells and donor serum, patient cells) may be advisable.

K3.000 When the patient has aplastic anemia, every effort should be made to complete tests as rapidly as possible to minimize the number of pretransplant blood transfusions.

K4.000 Unrelated donors.

K4.100 The donor should give his informed consent before blood is taken for typing and before the donor is placed on a list of donors available to be called.

K4.200 Donor records should be maintained so that donors can be rapidly retrieved according to HLA type.

K4.300 Laboratories should have a mechanism in place for resolving any tissue typing discrepancies that may occur between laboratories.

L – PLATELET AND GRANULOCYTE TRANSFUSION

L1.000 HLA Typing.

L1.100 The patient and members of his immediate family should be typed for HLA-A and B antigens.

L1.200 Typing for HLA-C, D, DR, DQ and DP is not necessary.

L2.000 The donor should give his informed consent before blood is taken for typing and before the donor is placed on a list of donors available to be called.

L2.100 Donor records should be maintained so that donors can be rapidly retrieved according to HLA type.

L3.000 Screening the sera of patients for lymphocytotoxic antibodies at intervals is an appropriate way to detect alloimmunization.

L4.000 Crossmatching.

L4.100 Lymphocytotoxic crossmatches are optional.

L4.200 Crossmatching by techniques which utilize donor platelets or granulocytes as the target cells is preferred.

M – DISEASE ASSOCIATION

M1.000 Complete HLA typing is an appropriate option.

M1.100 Typing may also be limited to all products of a single or limited number of HLA loci.

M2.000 Typing for a Single Antigen (e.g., HLA-B27).

M2.100 Cell controls must be tested on each batch of typing-trays.

M2.110 The control cells must include at least two cells known to express the specified antigen.

M2.120 The control cells must also include two cells for each cross-reacting antigen which might be confused with the specific antigen.

M2.130 The control cells must also include at least two cells lacking the specific and crossreacting antigens.

M2.200 Serum controls must be tested at the time of typing.

M2.210 Serum controls must include a positive and negative control.

M2.220 Serum controls should also include two sera for each antigen which crossreacts with the specified antigen (if available).

M2.300 Sera to define each antigen must meet requirements of Sections E or F as appropriate.

N – PARENTAGE TESTING

N1.000 Parentage testing must be restricted to laboratories whose Director fulfills the general Director qualifications (B1.000) and in addition is qualified by advanced training and/or experience in parentage testing.

N1.100 The competency of the technical staff in relation to parentage testing must be the responsibility of the Director.

N1.200 The laboratory Director and technical staff performing parentage testing must participate in continuing education relative to the field of parentage testing.

N1.300 A qualified individual must be available for legal testimony in the case, as needed.

N2.000 Laboratories utilizing genetic systems in addition to HLA must be able to document expertise and/or accreditation in those systems.

N2.100 An accredited laboratory may engage another laboratory to perform genetic testing for systems not used by the primary laboratory. In that event, the subcontracting laboratory and that portion of the testing for which it bears responsibility must be noted in the report (see N7.000).

N3.000 Subject Identification.

N3.100 Evidence for verifiable means of identification for subjects must be recorded at the time the blood sample is taken.

N3.200 Recommended evidence includes photographs, fingerprints and the number(s) of identification cards displaying the subject's picture (e.g., drivers license).

N3.300 Specimens received from an outside collecting facility must also have a means for positive identification unless this requirement has been waived by mutual consent of the individuals involved.

N3.400 A record must be kept at the testing facility of all identifying information including, but not limited to, name, relationship, race, place and date of collection of sample. Information about each individual must be verified by the signature of that person or the guardian.

N3.500 The date of birth of the child and recent transfusion history (past three months) of each individual to be tested must be recorded.

N4.000 Sample Identification.

N4.100 Each tube must be labeled immediately prior to or following collection of the sample to avoid mix-up of samples.

N4.200 The label must include the full name of the subject, the date and the initials of the blood drawer.

N4.300 The phlebotomist's name must be part of the permanent record.

N4.400 A record of the "chain of custody" of the sample must be maintained.

N5.000 HLA Testing Requirements for Parentage Testing.

N5.100 Each test sample must be plated on two separate trays or tray sets each containing a minimum of one monospecific or two multispecific sera defining each HLA-A and B locus antigen tested. The sera defining a particular specificity should be from different donors. The trays must be read independently.

N6.000 Calculations.

N6.100 Computer assisted analyses must be reviewed, verified and signed by the Supervisor and/or Laboratory Director before issue.

N6.200 The computer program which is utilized for analyses must be documented.

N6.300 If only manual calculations are done, they must be done in duplicate.

N6.400 Gene and haplotype frequencies should have been obtained from examination of populations of adequate size.

N7.000 Reports.

N7.100 Each report must be released only to authorized individuals and must contain:

N7.110 The name of each individual tested and the relationship to the child.

N7.120 The racial origin(s) assigned by the laboratory to the mother and alleged father(s) for the purpose of calculation.

N7.130 The phenotypes established for each individual in each genetic system examined.

N7.140 A statement as to whether or not the alleged father can be excluded. When there is no exclusion, the report must contain:

N7.141 The individual Paternity Index for each genetic system reported.

N7.142 The cumulative Paternity Index.

N7.143 The probability of paternity expressed as a percentage. The prior probability(ies) used to calculate the probability of paternity must be stated.

N7.144 Other mathematical or verbal expressions are optional. If they are included in the report, such expressions should be defined and explained.

N7.150 If the results are inconclusive, an explanation as to the nature of the problem.

N7.160 The signature of the laboratory Director.

P – NUCLEIC ACID ANALYSIS

The nucleic acid analysis standards apply to histocompatibility testing.

P1.000 Restriction Fragment Length Polymorphism (RFLP).

P1.100 Restriction Endonucleases.

P1.110 Enzymes must be stored and utilized under conditions recommended by the manufacturer (i.e. storage temperature, test temperature, buffer) to ensure proper DNA digestion.

P1.120 It should be documented that each lot of enzyme produces human DNA polymorphism of known sizes prior to analysis of results.

P1.130 When DNA is digested for analysis, human DNA which will produce polymorphism of known sizes must also be digested to ensure complete endonuclease digestion.

P1.200 Probes.

P1.210 Each DNA probe utilized should be validated by family studies demonstrating Mendelian inheritance of the polymorphism detected and by extensive population studies.

P1.220 The probe should be used in the form as reported in the scientific literature and as was used to determine the inheritance pattern and population distribution of the polymorphism.

P1.300 DNA Extraction.

P1.310 DNA should be purified by a standard method that has been reported in the scientific literature and validated in the laboratory.

P1.320 If the DNA is not used immediately after purification, suitable methods of storage should be available that would protect the integrity of the material.

P1.330 DNA must be intact and not degraded.

P1.400 Electrophoresis.

P1.410 Size markers of known sequences that give discrete electrophoretic bands that span and flank the entire range of the DNA system being tested must be included in the electrophoretic run. The known human control DNA used to determine that complete endonuclease digestion was achieved, must also be included in each electrophoretic run as a control.

P1.420 Equal amounts (mg/ml) of DNA must be loaded per lane.

P1.430 A photograph of the ethidium bromide pattern resulting from the electrophoretic separation should be kept for each run.

P1.500 Prehybridization, Hybridization, Autoradiography.

P1.510 Prehybridization, hybridization, autoradiography must be carried out under empirically determined conditions of concentration, temperature and salt concentration which are determined by the nature of the probe.

P1.520 Stringency conditions should be selected to minimize the possibility of cross-hybridization.

P1.530 Probes should be labeled by a method appropriate for the probe in use. Nick translation, hexamer priming, end labeling or avidin biotin may be appropriate.

P1.540 Each probe used should give a signal adequate to detect a single copy gene. Whenever possible, locus-specific probes should be used.

P1.550 Re-probing of the same membrane should be performed only after complete stripping of the first probe.

P1.600 Analysis

P1.610 Only autoradiographs or membranes that reveal the appropriate patterns of the human control DNA and size markers should be analyzed.

P1.620 Each autoradiograph or membrane should be read independently by two or more individuals.

P1.630 The laboratory report for each fragment detected **should** specify the probe, restriction endonuclease used, fragment size (k.b.) and the chromosomal location as defined by the International Human Gene Mapping Workshop.

P2.000 Amplification-based Typing

P2.1000 Amplification

P2.1100 Laboratory Design.

Use of physical and/or biochemical barriers to prevent DNA contamination (carry-over) is required. Pre-amplification procedures must be performed in a dedicated work area that excludes amplified DNA that has the potential to serve as a template for amplification in the HLA typing assays (e.g., PCR product, plasmids containing HLA genes). Physical separation and restricted traffic flow is recommended. Use of a static air hood or a Class II biological safety cabinet is recommended.

Biochemical procedures can be used to inactivate amplified products.

P2.1200 Other pre-amplification physical containment. Physical containment must include use of dedicated lab coats, gloves and disposable supplies. Frequent cleaning with dilute acid or bleach and/or UV treatment of work surfaces is recommended.

P2.1300 Equipment and Reagents.

P2.1310 Equipment.

P2.1311 Use of dedicated equipment for pre-amplification procedures is recommended.

P2.1312 Use of dedicated pipettors is required. Positive displacement pipettes or filter-plugged tips are recommended.

P2.1313 Thermal cycling instruments must precisely and reproducibly maintain the appropriate temperature of samples. Accuracy of temperature control for samples should be verified on a regular basis.

P2.1320 Reagents.

P2.1321 All reagents (solutions containing one or multiple components) utilized in the amplification assay must be dispensed in aliquots for single use or reagents can be dispensed in aliquots for multiple use if documented to be free of contamination at each use. When reagents are combined to create a master mix, it is recommended that one critical component (e.g. Mg++) be left out of the aliquot.

P2.1322 Reagents (e.g., chemicals, enzymes) must be stored and utilized under conditions recommended by the manufacturer (i.e., storage temperature, test temperature, buffer, concentration). Reagents used for amplification must not be exposed to post-amplification work areas. The appropriate performance of each lot of reagent must be documented before results using these reagents are reported.

P2.1323 For commercial kits, the source, lot number, expiration date, and storage conditions must be documented. Reagents from different

lots of kits must not be mixed. Each laboratory is responsible for the accuracy of typing. One possible approach for quality control is to test each reagent with a positive and negative control.

P2.1324 Primers must be stored under conditions that maintain specificity and sensitivity.

P2.1325 Methods that utilize two consecutive steps of logarithmic amplification are especially susceptible to errors related to PCR carryover (contamination) and special attention must be paid to containment of amplified products (e.g., physical separation, work flow and enhanced contamination monitoring). Standard 2.1100 applies to all components of the second amplification except template. Addition of the template for the second amplification must be physically separated from the pre-amplification work area and the post-amplification work area. Use of pipettors dedicated to each work area (i.e. first amplification, second amplification and analysis) is required.

P2.1400 Amplification templates

P2.1410 Specimens must be stored under conditions that do not result in artifacts or inhibition of the amplification reaction. Specimens must not be exposed to post-amplification work areas.

P2.1420 Nucleic acids should be prepared by a standard method that has been validated in the laboratory.

P2.1430 DNA or cDNA (from RNA templates) is satisfactory. DNA from any nucleated cells or RNA from any cells expressing the HLA product may be used. If RNA is used, appropriate positive controls for reverse transcription must be included.

P2.1440 Nucleic acids must be prepared and stored in a manner which does not result in artifacts or inhibition of the amplification reaction. The acceptable range for the amount of target must be specified and validated.

P2.1500 Primers.

P2.1510 The specificity and sequence of primers must be defined. The HLA locus and allele(s) must be defined.

P2.1520 Conditions which influence the specificity or quantity of amplified product must be demonstrated to be satisfactory for each set of primers.

P2.1530 Reference material should be used to test and periodically reconfirm the specificity and product quantity of each lot of primers.

P2.1600 Contamination.

P2.1610 Nucleic acid contamination must be monitored. Controls must be tested using the method that is routinely used to detect HLA types.

P2.1611 Negative controls (no nucleic acid) must be included in each amplification assay. Another negative control might include open tubes in the work area.

P2.1612 In order to minimize the detection of minor contaminants and the occurrence of stochastic fluctuation the number of cycles should be set at a level sufficient to detect the target nucleic acid but insufficient to detect small amounts (e.g., <10 molecules) of contaminating template.

P2.1613 Routine wipe tests of pre-amplification work areas must be performed. If amplified product is detected, the area must be cleaned to eliminate the contamination and measures must be taken to prevent future contamination.

P2.1700 Controls.

P2.1710 The quantity of specific amplification products must be monitored (e.g., gel electrophoresis, hybridization).

P2.1720 Criteria for accepting or rejecting an amplification assay must be specified.

P2.1730 If presence of an amplified product is used as the end result, controls must be included to detect amplification failure in every amplification mixture. Amplification specificity must be monitored on a periodic basis.

P2.2000 Amplified Product (Nucleic Acid Targets)

P2.2100 Variation in the amount of amplified product must be monitored (e.g., hybidization with a consensus probe, gel electrophoresis). The acceptable range for the amount of available target must be specified.

P2.3000 Oligonucleotide Probes

P2.3100 HLA locus and allele(s) must be defined for each probe and template combination. Positive or negative probe hybridization must be defined for each probe with all possible combinations of alleles that are recognized by the W.H.O. provided that nucleotide sequences are readily available.

P2.3200 Probes must be stored under conditions which maintain specificity and sensitivity.

P2.3300 Probes must be utilized under empirically determined conditions that achieve the defined specificity. The specificity should be demonstrated and maintained for each lot of probe. Each lot of probes should be tested for specificity and product quantity using reference material under optimized conditions and reconfirmed periodically.

P2.3400 Hybridization must be carried out under empirically determined conditions that achieve the defined specificity.

P2.3500 The specificity of hybridization should be confirmed using positive and negative controls for hybridization with each probe. The controls should be capable of detecting cross-hybridization with closely related sequences.

P2.3600 Reuse of nucleic acids (probes or targets) bound to solid supports should only be undertaken after demonstrating that previous signals are no longer detectable.

P2.3700 Reuse of nucleic acids in solution (probes or targets) should only be undertaken with controls to ensure that the sensitivity and specificity of the assay are unaltered.

P2.3800 Incubators and water baths must be monitored for precise and accurate temperature maintenance every time the assay is performed.

P2.4000 Labeling of nucleic acids and detection

P2.4100 The specificity and sensitivity of the labeling and detection method must be established and reproducible.

P2.4200 The specificity and sensitivity must be maintained for each lot of reagents (e.g., antibodies, probes, indicator molecules).

P2.4300 Enzymes must be stored and utilized under conditions recommended by the manufacturer (i.e., storage temperature, test temperature, buffer, concentration) to ensure correct enzymatic activity. The enzymatic activity of each lot should be confirmed before use.

P2.5000 Analysis

P2.5100 Acceptable limits of signal intensity must be specified for positive and negative results. If these are not achieved, corrective action is required.

P2.5200 The method of assignment of types must be designated.

P2.5300 Two independent interpretations of primary data are recommended.

P2.5400 Reports must designate the type of assay (e.g., PCR/oligonucleotide), indicate the HLA locus, and define each type using W.H.O. nomenclature for alleles.

P2.5500 A permanent record of primary data must be retained for 2 years.

P2.6000 Nucleotide Sequencing.

P2.6100 Sequencing Templates.

Standards in P2.1400 must be followed for preparation of templates.

P2.6110 Templates must have sufficient specificity (e.g., locus or allele-specificity), quantity and quality to provide interpretable primary sequencing data. The method for preparing templates must reliably generate appropriate length sequencing templates that are free of

inhibitors of subsequent reactions (e.g., primer extension) and free of contaminants that cause sequencing artifacts. Methods must ensure that preparation of templates does not alter the accuracy of the final sequence (e.g., mutations created during cloning, preferential amplification).

P2.6120 Reagents used in preparation of templates (e.g., enzymes, biochemicals) must be stored and utilized under conditions recommended by the manufacturer. The appropriate performance of each lot must be documented before results of tests using these reagents are reported.

P2.6200 Methods Utilizing Primer Extension.

P2.6210 The specificity and general knowledge of the target sequence must be defined. The HLA locus and allele(s) must be defined.

P2.6220 Primers must be used under empirically determined conditions that achieve the defined specificity of amplification. The amplification conditions must be demonstrated by the laboratory to achieve defined specificity and must yield adequate quantity of specific product. Each lot of primer should be tested for specificity and product quantity using reference material (e.g. DNA) under routine conditions and reconfirmed periodically.

P2.6230 Conditions for primer extension (e.g., polymerase type, polymerase concentration, primer concentration, concentration of nucleoside triphosphates, concentration of terminators) must be appropriate for the template (e.g., length of sequence, GC content).

P2.6240 The specificity and sensitivity of the labeling and detection methods must be documented (e.g., demonstrating correct signal strength for a control sequence) in the laboratory before results are reported.

P2.6250 Satisfactory performance of each lot of reagent (e.g., nucleotides, enzymes) must be documented before results using these reagents are reported. Reagents must be stored under conditions that maintain optimal performance.

P2.6300 Electrophoresis.

P2.6310 A sequencing standard must be run on every gel. The laboratory must establish scientifically and technically sound criteria for accepting each gel and each lane of a gel.

P2.6320 A permanent record of each electrophoretic run (e.g., electronic file, hard copy) must be retained for at least two years.

P2.6330 Satisfactory performance of each lot of reagents that influence the quality and accuracy of sequencing data of the gel (e.g., acrylamide, buffer and salt concentration) should be documented before results using these reagents are reported. Acceptable electrophoretic conditions (e.g., temperature, voltage, duration) must be established. Conditions should be recorded for each run. Reagents must be stored under conditions that maintain acceptable performance.

P2.6400 Nucleotide assignments

P2.6410 Criteria for acceptance of primary data must be established (e.g., correct assignments for nonpolymorphic positions, certain region of sequence, criteria for peak intensity, baseline fluctuation, signal-to-noise ratio and peak shapes). Validation might include sequencing of representatives of all polymorphic motifs that are frequently encountered in the routine sample population to detect sequence-specific artifacts. Sequencing of both strands of at least one representative of each polymorphic motif is recommended during validation. Established sequence-specific characteristics should be documented and utilized in routine interpretation of data.

P2.6420 Routine sequence assignments should be based on analysis of sequence data from complementary strands of DNA unless it is documented that the sequencing method consistently yields accurate sequence assignments using data from only one strand of DNA. If assignments are routinely based upon data from one strand of DNA, periodic confirmation of complementary strands is recommended. If base assignments are frequently difficult to interpret, routine sequencing of both strands is recommended. If a sequence suggests a novel allele or a rare combination of alleles, the sequences of both strands must be determined.

P2.6430 A scientifically sound and technically sound method must be established for interpretation, acceptance, and/or rejection of sequences from regions which are difficult to resolve (e.g., compression, ends).

P2.6440 Two independent interpretations of the primary data are recommended.

P2.6450 Automated systems and computer programs for nucleotide assignments must be validated prior to use.

P2.6500 Allele Assignments

P2.6510 HLA locus and alleles must be defined for each template/primer combination. Each unknown sequence must be compared with the sequences of all alleles that are recognized by the W.H.O. provided that the nucleotide sequences are readily available (i.e., in a locus-specific alignment in conjunction with the W.H.O. Nomenclature Committee for Factors of the HLA System which appears periodically in the public domain such as *Tissue Antigens,* the ASHI Web Pages or *Human Immunology*. Databases of sequences must be accurate and conform to the most recent compilation of sequences published in conjunction with the W.H.O.

P2.6520 Ambiguous combinations of alleles should be defined for each template/primer combination

P2.6530 Methods must ensure that sequences contributed by amplification primers are not considered in the assignment of alleles.

P2.6540 Two independent assignments of alleles are recommended.

P2.6550 Automated systems and computer programs for allele assignments must be validated prior to use.

P2.6560 Reports must designate the type of assay, HLA locus, and define each type using W.H.O. Nomenclature for alleles. The laboratory must maintain records that define the sequence database utilized to interpret the primary data. This database must be updated periodically. If a determined sequence is ambiguous (i.e. more than one possible interpretation of available data) the report must indicate all possible allelic combinations.

P2.7000 Restriction Fragment Length Polymorphism of Amplified Products

P2.7100 Restriction endonucleases.

P2.7110 HLA locus and allele(s) must be defined for each RFLP type.

P2.7120 Enzymes must be stored and utilized under conditions recommended by the manufacturer (i.e., storage temperature, test temperature, buffer, concentration) to ensure correct enzymatic activity. The appropriate performance of each lot of enzyme must be documented before results using these reagents are reported.

P2.7130 When amplified DNA is digested, controls of amplified DNA which will produce fragments of known sizes must also be digested in parallel to monitor complete digestion.

P2.7200 Electrophoresis.

P2.7210 Size markers of known sequence that produce discrete electrophoretic bands spanning and flanking the entire range of expected fragment sizes must be included in every run.

P2.7220 The amount of DNA/lane must not alter the rate of migration with respect to the migration of controls.

P2.7230 A permanent record (e.g., photograph, image) of each electrophoretic run must be retained as defined in C5.1000.

P2.7240 Amplified DNA should be incubated without restriction enzyme and analyzed by gel electropheresis to monitor marker integrity.

P2.7300 Analysis.

P2.7310 Acceptable limits of signal intensity must be specified for positive and negative results. If these are not achieved, corrective action is required.

P2.7320 Appropriate migration patterns of control DNA and size markers are required.

P2.7330 The method of assignment of HLA types must be designated.

P2.7340 Two independent interpretations of primary data are recommended.

P2.7350 Reports must designate the type of assay (e.g., PCR/RFLP), indicate the HLA locus, and define each HLA type using W.H.O. nomenclature for alleles.

P2.8000 Typing Using Sequence-Specific Amplification

P2.8100 HLA locus and allele(s) must be defined for each primer combination. Positive or negative amplification must be defined for each primer mixture with all possible combinations of alleles that are recognized by the W.H.O. provided that nucleotide sequences are readily available.

P2.8200 Each amplification reaction must include procedures to detect technical failures (e.g., an internal control such as additional primers or templates that produce a product that can be distinguished from the typing product).

P2.8300 In each amplification assay (i.e. set up of amplification mixtures for one or more samples) controls should be used to detect contamination with previously amplified products (e.g., a special primer pair internal to all amplification products or a combination of primers to detect any DNA that could confound the typing result).

P2.8400 Primers must be utilized under empirically determined conditions that achieve the defined specificity for templates used in routine testing. Each set of primers must be tested for amplification specificity and product quantity using reference cells under optimized conditions. The frequency of testing each primer set must ensure that all primer pairs have appropriate sensitivity and specificity of amplification. The specificity and sensitivity must be maintained in heterozygous samples.

P2.8500 The specificity and sensitivity of the detection method must be established and reproducible.

P2.8600 Analysis

P2.8610 Acceptable qualitative limits of signal intensity must be specified for positive and negative results. If these are not achieved, corrective action is required.

P2.8620 The method of assignment of types must be designated.

P2.8630 Two independent interpretations of primary data are recommended.

P2.8640 Reports must designate the type of assay (e.g., SSP), indicate the HLA locus, and define each type using W.H.O. nomenclature for alleles.

P2.8650 A permanent record of primary data must be retained for 2 years.

P2.9000 Other Methods

P2.9100 If alternate methods (e.g., SSCP, heteroduplex, DGGE) are used for HLA typing, established procedures must be defined and must include sufficient controls to ensure accurate assignment of types for every sample. All relevant standards from the above sections should be applied.

P2.9200 Automated systems and computer programs must be validated prior to use and tested routinely for accuracy and reproducibility of manipulations.

Q – FLOW CYTOMETRY

These standards apply to histocompatibility testing and leucocyte phenotyping by flow cytometry.

Q1.000 Instrument Standardization/Calibration.

Q1.100 An optical standard, consisting of latex beads or other uniform particles, shall be run to insure proper focusing and alignment of all lenses in the path for both the exciting light source and signal (light scatter, fluorescence, etc.) detectors.

Q1.110 The optical standard shall be run each time the instrument is turned on and any time maintenance, adjustments or sample problems likely to have altered optical alignment (obstruction of fluidics) occur during operation.

Q1.120 The results of optical focusing/alignment must be recorded in a daily quality control log.

Q1.130 A threshold value for acceptable optical standardization must be established for all relevant signals for each instrument and the focusing procedure repeated until these values are achieved or surpassed.

Q1.140 In the event a particular threshold value cannot be attained, a written protocol for instituting corrective action must be available. This protocol should include appropriate corrective actions including clear guidelines describing when a service call is warranted.

Q1.200 A fluorescent standard for each fluorochrome to be used, shall be run to insure adequate amplification of the fluorescent signal(s) on a day-to-day basis.

Q1.210 This standard may be incorporated in the beads or other particles used for optical standardization or may be a separate bead or fixed cell preparation.

Q1.220 The fluorescent standard must be run each time the instrument is turned on and any time maintenance, adjustments or sample problems likely to have altered the gain or high voltage settings (e.g. obstruction of fluidics) occur during operation.

Q1.230 The results of fluorescent standardization shall be recorded in a daily quality control log.

Q1.240 In the event that acceptable fluorescence separation cannot be attained, a written protocol for instituting corrective action must be available. This protocol should include appropriate corrective action including clear guidelines describing when a service call is warranted.

Q1.300 If performing analyses that require the simultaneous use of two or more fluorochromes, an appropriate procedure must be used to compensate for "spill over" into the other fluorescence detectors.

Q1.400 For laser based instruments, the current input (amps) and laser light output (milliwatts), at the normal operating wavelength measured after the laser is peaked and normal operating power set, must be recorded as part of a daily quality control record.

Q2.000 Flow Cytometric Crossmatch Technique

Q2.100 A multi-color technique is highly recommended. However, if a single color technique is used, the purity of the isolated cell population must be documented and should be of sufficient purity to define the population for analysis.

Q2.110 The binding of human immunoglobulin should be assessed with a fluorochrome labelled (e.g., fluorescein) F(ab')2 anti-human IgG.

Q2.120 Binding of antibody to T cells, B cells and/or monocytes should be positively confirmed with a differently labelled (e.g., phycoerythrin) monoclonal antibody that detects the corresponding cluster designated antigen (e.g., CD3 for T cells, CD19 or CD20 for B cells and CD14 for monocytes).

Q2.130 Multicolor staining of other immunoglobulin classes and target cells may also be justified.

Q2.140 Each laboratory should establish and document the optimum serum/cell ratio i.e., a standard number of cells to a fixed volume of serum.

Q2.200 Controls.

Q2.210 The normal human serum control should be from a non-alloimmunized and otherwise healthy individual and must be screened by flow cytometry to insure lack of reactivity against human lymphocytes.

Q2.220 The positive control should be human serum containing antibodies of the appropriate isotype, specific for the HLA antigens or any other alloantigens deemed to be important for detection in the crossmatch. Positive controls should react with lymphocytes of all humans.

Q2.230 The anti-human immunoglobulin reagent should be titered to determine the dilution with optimal activity (signal to noise ratio). If a multicolor technique is employed, the reagent must not demonstrate crossreactivity with the other immunoglobulin reagents used to mark the cells.

Q2.240 Regardless of the method used for reporting raw data (mean, median, mode channel shifts or quantitative fluorescence measurements), each lab must establish its own threshold for discriminating positive reactions. Any significant change in protocol, reagents or instrumentation requires repeat determination of the positive threshold.

Q2.300 Interpretation

Q2.310 Each laboratory must define the criteria used to define positive and negative crossmatches.

Q3.000 Immunophenotyping By Flow Cytometry

Q3.100 Terminology used must be defined and/or conform to nomenclature recommended/approved by the most recent International Workshop of Differentiation Antigens of Human Leucocytes or other appropriate scientific organizations.

Q3.200 Cell Preparation.

Q3.210 The method used for cell preparation should be documented to yield appropriate preparations of viable cells.

Q3.220 The viability of cell preparations should be recorded and should exceed the laboratory's established minimum standards for each procedure used.

Q3.230 For internal labelling, the method used to allow fluorochrome labelled antibodies to penetrate the cell membrane must be documented to be effective.

Q3.300 Labeling of Specimens.

Q3.310 Specificity controls, consisting of appropriate cell types known to be positive for selected standard antibodies must be run within laboratory-defined intervals sufficiently short to assure the proper performance of reagents.

Q3.320 A negative reagent control(s) shall be run for each test cell preparation. This control should consist of monoclonal antibody(ies) of the same species and subclass and should be prepared/purified in the same way as the monoclonal(s) used for phenotyping.

Q3.330 For indirect labelling, the negative control reagent should be an irrelevant primary antibody, if available, and in all cases, the same secondary antibody(ies) conjugated with the same fluorochrome(s) used in all relevant test combinations.

Q3.340 For direct labelling, the negative control reagent should be an irrelevant antibody conjugated with the same fluorochrome and at the same fluorochrome:protein ratio used in all relevant test combinations.

Q3.350 Whether analyzed directly or fixed prior to analysis, labelled cells must be analyzed within a time period demonstrated by the laboratory to avoid significant loss of any cell subpopulation or total cell numbers. Control samples must be analyzed within the same period after staining as the test samples.

Q3.360 If analysis will be based on a population of cells selected by flow cytometry "gating" on size or density parameters, or selected by depletion or enrichment techniques, control stains must be run for each test individual to detect the presence of contaminating cells in the selected population. (e.g., Monocyte contamination of 'lymphocytes' gated by forward angle or forward angle vs 90° light scatter must be detected with a monocyte specific marker antibody.

Q3.370 Conclusions about abnormal proportions or abnormal numbers of cells bearing particular internal or cell surface markers must only be drawn in comparison with local 'control' data obtained with the same instrument, reagents and techniques.

Q3.380 Determination of percent positives must take into consideration the results of the negative control reagent. However, when clearly defined positive and negative populations are evident in the test sample, it may be appropriate to adjust the threshold based on the test sample.

Q3.400 Reagents

Q3.410 The specificity of monoclonal antibodies shall be verified by published and/or manufacturer's documentation and whenever possible verified locally through tests with appropriate control cells prepared and tested by the same method employed in the laboratory's test sample analysis.

Q3.420 The quantities of reagents used for each test sample must be determined by the manufacturers or from published data and whenever possible should be verified locally by appropriate titration procedures.

Q3.430 Reagents must be stored according to manufacturers' instructions or according to conditions verified to maintain stability by documented local tests.

Q3.440 Monoclonal antibodies which have been reconstituted from lyophilized powder form for storage at 4°C should be centrifuged according to the manufacturer's instructions or locally documented procedures to remove microaggregates prior to use in preparation of working stains.

Q4.000 HLA Typing By Flow Cytometry (e.g., HLA B27)

Q4.100 Terminology used must be defined and/or conform to nomenclature recommended/approved by the most recent W.H.O. nomenclature committee meeting.

Q4.200 Cell Preparation.

Q4.210 The method used for cell preparation should be documented to yield appropriate preparations of viable cells.

Q4.220 The viability of cell preparations should be recorded and should exceed the laboratory's established minimum standards for each procedure used.

Q4.2300 Labelling of specimens.

Q4.2310 A negative reagent control(s) shall be run for each test cell preparation. This control should consist of monoclonal antibody(ies) of the same species and subclass and should be prepared/purified in the same way as the monoclonal(s) used for phenotyping. Negative reagent controls should consist of:

Q4.2311 For indirect labelling, an irrelevant primary antibody, if available, and in all cases, the same secondary antibody(ies) conjugated with the same fluorochrome(s) used in all relevant test combinations.

Q4.2312 For direct labelling, an irrelevant antibody conjugated with the same fluorochrome and at the same fluorochrome: protein ratio used in all relevant test combinations.

Q4.2320 Whether analyzed directly or fixed prior to analysis, labelled cells must be analyzed within a time period demonstrated by the laboratory to avoid significant change in test results. Control samples must be analyzed within the same period after staining as the test samples.

Q4.3000 Reagents.

Q4.3100 The specificity of monoclonal antibodies shall be verified through tests with appropriate control cells prepared and tested by the same method employed in the laboratory's test sample.

Q4.3200 Cell controls must be tested for each batch of monoclonal antibodies received.

Q4.3210 The control cells must include at least five cells known to express the specified antigen.

Q4.3220 The control cells must also include two cells for each cross-reacting antigen which might be confused with the specific antigen.

Q4.3230 The control cells must also include at least two cells lacking the specific and crossreacting antigens.

Q4.3300 The quantities of reagents used for each test sample must be determined by the manufacturers or from published data and whenever possible should be verified locally by appropriate titration procedures.

Q4.3400 Reagents must be stored according to manufacturer's instructions or according to conditions verified to maintain stability by documented local tests.

Q4.3500 Monoclonal antibodies which have been reconstituted from lyophilized powder form for storage at 4 degrees centigrade should be centrifuged according to the manufacturer's instructions or locally documented procedures to remove microaggregates prior to use in preparation of working stains.

Q4.3600 A single monoclonal antibody may be used to define an antigen provided its monospecificity has been sufficiently verified by local testing.

Q4.3700 Minimum reactivity for assignment of a positive reaction must be established by the laboratory.

Q4.3800 If the monoclonal antibody(ies) is (are) known or found to react with antigens other than the one specified, a written protocol must explain how its presence or absence is finally determined.

R – ENZYME-LINKED IMMUNO SORBENT ASSAY (ELISA)

R1.000 Instrument Standardization/Calibration.

R1.100 The ELISA reader.

R1.110 The light source and filter must produce the intensity and wavelength of light required for the test system.

R1.120 Precise movement of the plate must be verified and recorded.

R1.130 Periodic calibration must be performed according to the instrument manufacturer's instructions and must be documented.

R1.200 Assays must be performed with calibrated dispensing instruments. Calibration must be routinely performed routinely and must be documented.

R1.300 Microplate washer performance must be checked monthly and acceptable performance documented.

R2.000 ELISA Technique.

R2.100 If commercial kits are used, the manufacturer's instructions must be followed unless the laboratory has performed and documented testing to support a deviation in technique or analysis.

R2.200 Reagents must be stored at the temperature and for no longer than the duration specified by the manufacturer.

R2.300 Each assay must contain a positive control, a negative control and reagent controls. The dilution of reagents and test specimens must be documented.

R2.400 Sample identity and proper plate orientation must be maintained throughout the procedure.

R2.500 The lot numbers and optical density values of the reference reagents and the controls must be recorded for each assay. These values must fall within acceptable limits for the assay to be valid.

R2.600 The volume and number of washes must be recorded for each assay.

R2.700 New lots of reagents must be validated by side-by-side testing with a lot known to give acceptable performance or by testing with test specimens of known reactivity.

HLA Alleles and Equivalent Serological Types

HLA-A Locus Alleles and Equivalent Serological Types

A*0101	A1
A*0102	A1
A*0103	Not defined
A*0104N	Null
A*0201	A2
A*0202	A2
A*0203	A203
A*0204	A2
A*0205	A2
A*0206	A2
A*0207	A2
A*0208	A2
A*0209	A2
A*0210	A210
A*0211	A2
A*0212	A2
A*0213	A2
A*0214	A2
A*0215N	Null
A*0216	A2
A*0217	A2
A*0218	A2
A*0219	Not defined
A*0220	A2
A*0221	A2
A*0222	A2
A*0224	A2
A*0225	A2
A*0226	Not defined
A*0227	Not defined
A*0228	Not defined
A*0229	A2
A*0230	Not defined
A*0301	A3
A*0302	A3
A*0303N	Null
A*0304	A3
A*1101	A11
A*1102	A11
A*1103	A11
A*1104#	A11
A*1105#	A11
A*2301	A23 (9)
A*2402	A24 (9)
A*2402102L	Low A24
A*2403	A2403
A*2404	A24 (9)
A*2405	A24 (9)
A*2406	A24 (9)
A*2407	A24 (9)
A*2408	A24 (9)
A*2409N	Null
A*2410	A9
A*2411N	Null
A*2413	A24 (9)
A*2414	A24(9)
A*2415	Not defined
A*2416#	Not defined
A*2417	Not defined
A*2418#	Not defined
A*2419#	Not defined
A*2501	A25 (10)
A*2502	A25 (10)
A*2601	A26 (10)
A*2602	A26 (10)
A*2603	A26 (10)
A*2604	A26 (10)
A*2605	A26 (10)
A*2606	A26 (10)
A*2607	A26 (10)
A*2608	A26 (10)
A*2609	Not defined
A*2610#	A10
A*2611N	Null
A*2612	Not defined
A*2901	A29 (19)
A*2902	A29 (19)
A*2903	Not defined
A*2904	Not defined
A*3001	A30 (19)
A*3002	A30 (19)
A*3003	A30 (19)
A*3004	A30 (19)
A*3006	Not defined
A*3007	Not defined
A*3101	A31 (19)
A*3102	Not defined
A*3103	Not defined
A*3104	A31 (19)
A*3201	A32 (19)
A*3202	A32 (19)
A*3203	Not defined
A*3301	A33 (19)
A*3303	A33 (19)
A*3304	Not defined
A*3401	A34 (10)
A*3402	A34 (10)
A*3601	A36
A*4301	A43
A*6601	A66 (10)
A*6602	A66 (10)
A*6603	A10
A*6801	A68 (28)
A*6802	A68 (28)
A*6803	A28
A*6804	Not defined
A*6805	Not defined
A*6806	Not defined
A*6807	Not defined
A*6808	A68 (28)
A*6809	Not defined
A*6901	A69 (28)
A*7401	A74 (19)
A*7402	A74 (19)
A*7403	A19
A*8001	A80

For description of serological pattern, see Table 9 of Schreuder et al., The HLA dictionary 1999: a summary of HLA-A, -B, -C, -DRB1/3/4/5, -DQB1 alleles and their association with serologically defined HLA-A, -B, -DR and -DQ antigens. Tissue Antigens 1999; 54:409-437. Reprinted with permission.

HLA-B Locus Alleles and Equivalent Serological Types

B*0702	B7
B*0703#	B703
B*0704	B7
B*0705	B7
B*0706	B7
B*0707	B7
B*0708#	Not defined
B*0709	B7
B*0710	Not defined
B*0711	B7
B*0712	Not defined
B*0713	Not defined
B*0801	B8
B*0802#	B8
B*0803	B8
B*0804#	Not defined
B*0805	Not defined
B*0806	B8
B*1301	B13
B*1302	B13
B*1303#	Not defined
B*1304#	Not defined
B*1401	B64 (14)
B*1402	B65 (14)
B*1403	Not defined
B*1404	Not defined
B*1405	Not defined
B*1501	B62 (15)
B*1502	B75 (15)
B*1503	B72 (70)
B*1504	B62 (15)
B*1505	B62 (15)
B*1506	B62 (15)
B*1507	B62 (15)
B*1508	B75 (15)
B*1509	B70
B*1510	B71 (70)
B*1511	B75 (15)
B*1512	B76 (15)
B*1513	B77 (15)
B*1514	B76 (15)
B*1515	B62 (15)
B*1516	B63 (15)
B*1517	B63 (15)
B*1518	B71 (70)
B*1519	B76 (15)
B*1520	B62 (15)
B*1521	B75 (15)
B*1522	B35
B*1523#	Not defined
B*1524#	B62 (15)
B*1525	B62 (15)
B*1526N	Null
B*1527	B62 (15)
B*1528	B15
B*1529	B15
B*1530	B62 (15)
B*1531	B75 (15)
B*1532	B62 (15)
B*1533	B15
B*1534	B15
B*1535	B15
B*1536	Not defined
B*1537#	Not defined
B*1538#	Not defined
B*1539	Not defined
B*1540	Not defined
B*1542	Not defined
B*1543	Not defined
B*1544	Not defined
B*1545	B62 (15)
B*1546	B72 (70)
B*1547	Not defined
B*1548	B62 (15)
B*1549	Not defined
B*1801	B18
B*1802	B18
B*1803	B18
B*1804	Not defined
B*1805	B18
B*1806#	B18
B*1807	Not defined
B*2701	B27
B*2702	B27
B*2703	B27
B*2704	B27
B*2705	B27
B*2706	B27
B*2707	B27
B*2708#	B2708
B*2709	B27
B*2710	B27
B*2711#	B27
B*2712#	Not defined
B*2713	B27
B*2714	Not defined
B*2715#	Not defined
B*3501	B35
B*3502	B35
B*3503	B35
B*3504	B35
B*3505	B35
B*3506	B35
B*3507	B35
B*3508	B35
B*3509	B35
B*3510	Not defined
B*3511	B35
B*3512	B35
B*3513	B35
B*3514	B35
B*3515#	B35
B*3516	Not defined
B*3517	B35
B*3518	B35
B*3519	B35
B*3520	B35
B*3521	Not defined
B*3522	Not defined
B*3523	Not defined
B*3524	Not defined
B*3525	Not defined
B*3526	Not defined
B*3527	B35
B*3701	B37
B*3702#	Not defined
B*3801	B38 (16)
B*3802	B38 (16)
B*3803#	B16
B*3901	B3901
B*3902	B3902
B*3903	B39 (16)
B*3904	B39 (16)
B*3905#	B16
B*3906	B39 (16)
B*3907	Not defined
B*3908	B39 (16)
B*3909	B39 (16)
B*3910	B39 (16)
B*3911	Not defined
B*3912	B39 (16)
B*3913	B39 (16)
B*3914	Not defined
B*3915	Not defined
B*3916	Not defined
B*4001	B60 (40)
B*4002	B61 (40)
B*4003	B40
B*4004	B40
B*4005	B4005
B*4006	B61 (40)
B*4007	Not defined
B*4008#	Not defined
B*4009	B61 (40)
B*4010#	B60 (40)
B*4011	B40
B*4012#	Not defined
B*4013	Not defined
B*4014	Not defined

B*4015	Not defined	B*4803	Not defined	B*5502	B55 (22)
B*4016	B61	B*4804	Not defined	B*5503#	Not defined
B*4018	Not defined	B*4805	B48	B*5504	B55 (22)
B*4019	Not defined	B*4901	B49 (21)	B*5505	B22
B*4020	Not defined	B*5001	B50 (21)	B*5507	B54 (22)
B*4101	B41	B*5002#	B45 (12)	B*5508	Not defined
B*4102	B41	B*5101	B51 (5)	B*5601	B56 (22)
B*4103	Not defined	B*5102	B5102	B*5602	B56 (22)
B*4201	B42	B*5103	B5103	B*5603#	B22
B*4202	B42	B*5104	B51 (5)	B*5604#	B56 (22)
B*4402	B44 (12)	B*5105	B51 (5)	B*5605	Not defined
B*4403	B44 (12)	B*5106#	B5	B*5701	B57 (17)
B*4404	B44 (12)	B*5107	B51 (5)	B*5702	B57 (17)
B*4405	B44 (12)	B*5108	B51 (5)	B*5703	B57 (17)
B*4406#	B44 (12)	B*5109	B51 (5)	B*5704	B57 (17)
B*4407	B44 (12)	B*5110	Not defined	B*5705	Not defined
B*4408#	B44 (12)	B*5111N	Null	B*5801	B58 (17)
B*4409#	B12	B*5112#	Not defined	B*5802	B58 (17)
B*4410	Not defined	B*5113	Not defined	B*5901	B59
B*4411	Not defined	B*5114	Not defined	B*6701	B67
B*4501	B45 (12)	B*5115	Not defined	B*7301	B73
B*4502	Not defined	B*5116	B52 (5)	B*7801	B78
B*4601	B46	B*5201	B52 (5)	B*7802	B78
B*4701	B47	B*5301	B53	B*7803	Not defined
B*4702#	Not defined	B*5302	Not defined	B*8101	B81
B*4703#	Not defined	B*5303	Not defined	B*8201#	Not defined
B*4801	B48	B*5401	B54 (22)		
B*4802	B48	B*5501	B55 (22)		

For description of serological pattern, see Table 9 of Schreuder et al., The HLA dictionary 1999: a summary of HLA-A, -B, -C, -DRB1/3/4/5, -DQB1 alleles and their association with serologically defined HLA-A, -B, -DR and -DQ antigens. Tissue Antigens 1999; 54:409-437. Reprinted with permission.

HLA-C Locus Alleles and Equivalent Serological Types

Cw*0102	Cw1	Cw*0604	Not defined	Cw*1205	Not defined
Cw*0103	Cw1	Cw*0701	Cw7	Cw*1206	Not defined
Cw*0202	Cw2	Cw*0702	Cw7	Cw*1301	Not defined
Cw*0203	Not defined	Cw*0703	Not defined	Cw*1402	Not defined
Cw*0302	Cw10 (w3)	Cw*0704	Cw7	Cw*1403	Not defined
Cw*0303	Cw9 (w3)	Cw*0705	Not defined	Cw*1404	Not defined
Cw*0304	Cw10 (w3)	Cw*0706	Cw7	Cw*1502	Not defined
Cw*0305	Not defined	Cw*0707	Not defined	Cw*1503	Not defined
Cw*0306	Not defined	Cw*0708	Not defined	Cw*1504	Not defined
Cw*0307	Cw3	Cw*0709	Not defined	Cw*1505	Not defined
Cw*0308	Not defined	Cw*0710	Not defined	Cw*1506	Not defined
Cw*0309	Not defined	Cw*0711	Not defined	Cw*1507	Not defined
Cw*0401	Cw4	Cw*0712	Not defined	Cw*1508	Not defined
Cw*0402	Cw4	Cw*0801	Cw8	Cw*1601	Not defined
Cw*0403	Not defined	Cw*0802	Cw8	Cw*1602	Not defined
Cw*0404	Not defined	Cw*0803	Cw8	Cw*1604	Not defined
Cw*0405	Not defined	Cw*0804	Not defined	Cw*1701	Not defined
Cw*0406	Not defined	Cw*0805	Not defined	Cw*1702	Not defined
Cw*0501	Cw5	Cw*0806	Not defined	Cw*1801	Not defined
Cw*0502	Cw5	Cw*1202	Not defined	Cw*1802	Not defined
Cw*0602	Cw6	Cw*1203	Not defined		
Cw*0603	Not defined	Cw*1204	Not defined		

Schreuder et al., The HLA dictionary 1999: a summary of HLA-A, -B, -C, -DRB1/3/4/5, -DQB1 alleles and their association with serologically defined HLA-A, -B, -DR and -DQ antigens. Tissue Antigens 1999; 54:409-437. Reprinted with permission.

HLA-DR Locus Alleles and Equivalent Serological Types

DRB1*0101	DR1
DRB1*0102	DR1
DRB1*0103	DR103
DRB1*0104	DR1
DRB1*0105	Not defined
DRB1*0106	Not defined
DRB1*0301	DR17 (3)
DRB1*0302	DR18 (3)
DRB1*0303	DR18 (3)
DRB1*0304	DR17 (3)
DRB1*0305	DR17 (3)
DRB1*0306	DR3
DRB1*0307	Not defined
DRB1*0308	Not defined
DRB1*0309	Not defined
DRB1*0310	Not defined
DRB1*0311	Not defined
DRB1*0312	Not defined
DRB1*0313	Not defined
DRB1*0401	DR4
DRB1*0402	DR4
DRB1*0403	DR4
DRB1*0404	DR4
DRB1*0405	DR4
DRB1*0406	DR4
DRB1*0407	DR4
DRB1*0408	DR4
DRB1*0409	DR4
DRB1*0410	DR4
DRB1*0411	DR4
DRB1*0412	Not defined
DRB1*0413	DR4
DRB1*0414	DR4
DRB1*0415#	DR4
DRB1*0416	DR4
DRB1*0417	DR4
DRB1*0418	Not defined
DRB1*0419	DR4
DRB1*0420	DR4
DRB1*0421	DR4
DRB1*0422#	DR4
DRB1*0423	DR4
DRB1*0424	DR4
DRB1*0425#	DR4
DRB1*0426	DR4
DRB1*0427	Not defined
DRB1*0428	DR4
DRB1*0429	DR4
DRB1*0430	Not defined
DRB1*0431	Not defined
DRB1*0432	Not defined
DRB1*0701	DR7
DRB1*0703	DR7
DRB1*0704	Not defined
DRB1*0801	DR8
DRB1*0802	DR8
DRB1*0803	DR8
DRB1*0804	DR8
DRB1*0805	DR8
DRB1*0806	DR8
DRB1*0807	DR8
DRB1*0808	Not defined
DRB1*0809#	DR8
DRB1*0810	DR8
DRB1*0811	DR8
DRB1*0812	DR8
DRB1*0813	Not defined
DRB1*0814	DR8
DRB1*0815	Not defined
DRB1*0816	DR8
DRB1*0817	DR8
DRB1*0818	Not defined
DRB1*0819	Not defined
DRB1*0820	Not defined
DRB1*0821	Not defined
DRB1*0901	DR9
DRB1*1001	DR10
DRB1*1101	DR11 (5)
DRB1*1102	DR11 (5)
DRB1*1103	DR11 (5)
DRB1*1104	DR11 (5)
DRB1*1105	DR11 (5)
DRB1*1106	DR11 (5)
DRB1*1107#	Not defined
DRB1*1108	DR11 (5)
DRB1*1109	DR11 (5)
DRB1*1110	Not defined
DRB1*1111#	Not defined
DRB1*1112	Not defined
DRB1*1113#	DR11 (5)
DRB1*1114	DR11 (5)
DRB1*1115	Not defined
DRB1*1116#	Not defined
DRB1*1117	Not defined
DRB1*1118	Not defined
DRB1*1119	Not defined
DRB1*1120#	DR11 (5)
DRB1*1121	DR11 (5)
DRB1*1122	Not defined
DRB1*1123	DR11 (5)
DRB1*1124	Not defined
DRB1*1125	DR11 (5)
DRB1*1126	DR11 (5)
DRB1*1127	DR11 (5)
DRB1*1128	Not defined
DRB1*1129	DR11 (5)
DRB1*1130	Not defined
DRB1*1131	Not defined
DRB1*1132	Not defined
DRB1*1133	Not defined
DRB1*1134	Not defined
DRB1*1135	Not defined
DRB1*1201	DR12 (5)
DRB1*1202	DR12 (5)
DRB1*1203	DR12 (5)
DRB1*1204#	Not defined
DRB1*1205	DR12 (5)
DRB1*1206	DR12 (5)
DRB1*1301	DR13 (6)
DRB1*1302	DR13 (6)
DRB1*1303	DR13 (6)
DRB1*1304	DR13 (6)
DRB1*1305	DR13 (6)
DRB1*1306	DR13 (6)
DRB1*1307	DR13 (6)
DRB1*1308	DR13 (6)
DRB1*1309	Not defined
DRB1*1310	DR13 (6)
DRB1*1311#	DR13 (6)
DRB1*1312#	DR6
DRB1*1313	Not defined
DRB1*1314	DR13 (6)
DRB1*1315	Not defined
DRB1*1316	DR13 (6)
DRB1*1317#	DR13 (6)
DRB1*1318	DR13 (6)
DRB1*1319#	Not defined
DRB1*1320	DR13 (6)
DRB1*1321	Not defined
DRB1*1322	Not defined
DRB1*1323	Not defined
DRB1*1324	Not defined
DRB1*1325	Not defined
DRB1*1326#	Not defined
DRB1*1327	DR13 (6)
DRB1*1328	Not defined
DRB1*1329	DR6
DRB1*1330	Not defined
DRB1*1331	Not defined
DRB1*1332	Not defined
DRB1*1333	Not defined
DRB1*1334	Not defined
DRB1*1401	DR14 (6)
DRB1*1402#	DR14 (6)
DRB1*1403	DR1403
DRB1*1404	DR1404
DRB1*1405	DR14 (6)
DRB1*1406#	DR14 (6)
DRB1*1407	DR14 (6)

DRB1*1408	Not defined
DRB1*1409	Not defined
DRB1*1410	Not defined
DRB1*1411#	DR14 (6)
DRB1*1412	DR14 (6)
DRB1*1413	DR14 (6)
DRB1*1414	DR14 (6)
DRB1*1415#	DR8
DRB1*1416#	DR6
DRB1*1417#	DR6
DRB1*1418	DR6
DRB1*1419#	DR14 (6)
DRB1*1420#	DR14 (6)
DRB1*1421#	DR6
DRB1*1422#	Not defined
DRB1*1423	Not defined
DRB1*1424	Not defined
DRB1*1425	Not defined
DRB1*1426	DR14 (6)
DRB1*1427	DR14 (6)
DRB1*1428	Not defined
DRB1*1429	DR14 (6)
DRB1*1430	Not defined
DRB1*1431	Not defined
DRB1*1432	Not defined
DRB1*1433	Not defined
DRB1*1501	DR15 (2)
DRB1*1502	DR15 (2)
DRB1*1503	DR15 (2)
DRB1*1504	DR15 (2)
DRB1*1505	DR15 (2)
DRB1*1506	DR15 (2)
DRB1*1507	Not defined
DRB1*1508	DR2
DRB1*1601	DR16 (2)
DRB1*1602	DR16 (2)
DRB1*1603	DR2
DRB1*1604	DR16 (2)
DRB1*1605	DR2
DRB1*1607	Not defined
DRB1*1608	Not defined
DRB3*0101	DR52
DRB3*0102	Not defined
DRB3*0103	Not defined
DRB3*0104	Not defined
DRB3*0105	Not defined
DRB3*0201	DR52
DRB3*0202	DR52
DRB3*0203	DR52
DRB3*0204	Not defined
DRB3*0205	Not defined
DRB3*0206	Not defined
DRB3*0207	DR52
DRB3*0208	DR52
DRB3*0301	DR52
DRB3*0302	DR52
DRB3*0303	Not defined
DRB4*0101	DR53
DRB4*0102	DR53
DRB4*0103	DR53
DRB4*0103102N	Null
DRB4*0104	Not defined
DRB4*0105	DR53
DRB4*0201N	Null
DRB4*0301N	Null
DRB5*0101	DR51
DRB5*0102	DR51
DRB5*0103	Not defined
DRB5*0104	Not defined
DRB5*0105	Not defined
DRB5*0106	Not defined
DRB5*0107	DR51
DRB5*0108N	Null
DRB5*0109	Not defined
DRB5*0110N	Null
DRB5*0202	DR51
DRB5*0203	Not defined
DRB5*0204	Not defined

For description of serological pattern, see Table 10 of Schreuder et al., The HLA dictionary 1999: a summary of HLA-A, -B, -C, -DRB1/3/4/5, -DQB1 alleles and their association with serologically defined HLA-A, -B, -DR and -DQ antigens. Tissue Antigens 1999; 54:409-437. Reprinted with permission.

HLA-DQ Locus Alleles and Equivalent Serological Types

DQB1*0201	DQ2	DQB1*0309	Not defined	DQB1*0605	DQ6 (1)
DQB1*0202	DQ2	DQB1*0401	DQ4	DQB1*0606	Not defined
DQB1*0203	DQ2	DQB1*0402	DQ4	DQB1*0607	Not defined
DQB1*0301	DQ7 (3)	DQB1*0501	DQ5 (1)	DQB1*0608	Not defined
DQB1*0302	DQ8 (3)	DQB1*0502	DQ5 (1)	DQB1*0609	DQ6 (1)
DQB1*0303	DQ9 (3)	DQB1*0503	DQ5 (1)	DQB1*0610	Not defined
DQB1*0304	DQ7 (3)	DQB1*0504	DQ5 (1)	DQB1*0611	DQ1
DQB1*0305	DQ8 (3)	DQB1*0601	DQ6 (1)	DQB1*0612	DQ1
DQB1*0306	DQ3	DQB1*0602	DQ6 (1)	DQB1*0613	Not defined
DQB1*0307	Not defined	DQB1*0603	DQ6 (1)	DQB1*0614	DQ6 (1)
DQB1*0308	Not defined	DQB1*0604	DQ6 (1)	DQB1*0615	Not defined

Schreuder et al., The HLA dictionary 1999: a summary of HLA-A, -B, -C, -DRB1/3/4/5, -DQB1 alleles and their association with serologically defined HLA-A, -B, -DR and -DQ antigens. Tissue Antigens 1999; 54:409-437. Reprinted with permission.